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The Journal of Laboratory and Clinical Medicine

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CLINICAL AND EXPERIMENTAL

INTERPRETATION OF RADIOISOTOPIC LIPID TOLERANCE CURVES

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The wave of radioactivity in the blood stream after the ingestion of triolein labeled with radioactive iodine is subjected to mathematical analysis in order to derive a theory, based on acceptable physiology, which will adequately describe the curves obtained. The single channel type of theory, in which the ingested lipid follows the same metabolic pathway, disagrees by a large factor with observed results. Double channel types of theories do agree with observations. In this type of theory, human chyle absorbed from the alimentary tract appears to comprise two separate fractions: an alpha fraction which comprises about 5 per cent and is cleared slowly (in the range of 9 hours) and a beta fraction which comprises about 95 per cent and is cleared rapidly (about 5 to 10 minutes). Rate constants are derived to describe the various processes for the rate of absorption, clearance, oxidation, and renal clearance. The evaluation of these various constants in patients with disordered lipid metabolism suggests that they may serve as useful guides in the evaluation of the cause and degrees of lipid disorder. Various physical alternatives of the double channel type of theory are illustrated.

THE assessment of lipid tolerance in man using I^{131}-labeled triolein was first described by Thannhauser and Stanley.[1] This type of investigation was extended by Hall and co-workers.[2] In this later work the patient ingests a test meal that contains 100 Gm. of dietary fat and 20 μc of I^{131}-labeled triolein. The subsequent wave of radioactivity in the blood stream is then followed up for several days by means of venous blood samples. Radioactivity in the blood which is in the form of free iodide is separated and subtracted from the total. In some cases, the urinary excretion of the I^{131} has been followed up for several days.

From St. Vincent's Hospital, Sydney, Australia.
Received for publication April 13, 1959.

The interpretation and significance of the various curves obtained from this investigation are discussed in this article.

THEORY OF LIPID TOLERANCE CURVES

A set of typical lipid tolerance curves of the type to be discussed, obtained in a normal patient, is shown in Fig. 1, *A*, *B*, *C*, and *D*.

Fig. 1, *A* shows the normal amount of radioactivity appearing in the circulation, expressed as a percentage of the administered dose. The results have been plotted semilogarithmically and are typified by a rapid rise to a maximum of about 10 per cent in 6 hours. The subsequent decrease is approximately exponential with a time for half-clearance of about 9 hours. The ascending part of the curve is clearly determined by the patient's absorption mechanisms, whereas the descending part is determined by metabolic processes.

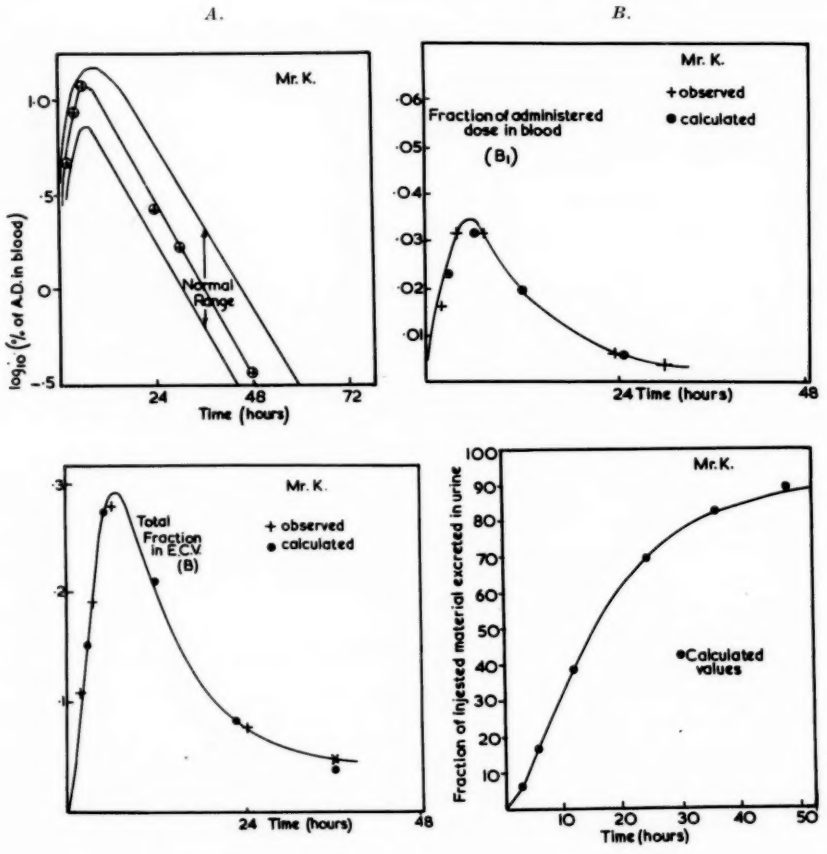


Fig. 1.—A set of results obtained in a patient with typically normal lipid metabolism. *A*, The results for total radioactivity against time, plotted on semilogarithmic scales. *B* and *C*, The results for the bound and free radioactivity. *D*, The radioactivity excreted in the urine.

When the blood radioactivity is separated into the bound and free fractions, the curves in Fig. 1, *B* and *C* are obtained. The bound iodide rises to a maximum in a few hours of a little under 5 per cent of the administered dose and again falls roughly exponentially with a half-value time of about 9 hours. In calculating the curve of the free iodide in Fig. 1, *C*, allowance has been made for the fact that the free I^{131} diffuses uniformly through the

iodide space which amounts in the male to 21 per cent of the body weight.[3] The maximum level of the free iodide occurs at approximately 12 hours and amounts to 30 per cent of the administered dose.

A typical curve of renal excretion of the radioactive marker is shown in Fig. 1, *D*. This should be, and is, simply related to the amount of I^{131} in the iodide space (B) by the equation

$$\frac{dU}{dt} = kB \qquad (1)$$

where k is the renal excretion constant.

We now require a theory, based on acceptable physiology, which will adequately describe the sort of curves shown in Fig. 1, *A* to *D*. It is generally accepted that fat is absorbed from the small intestine via the lymphatic route and is discharged as chyle into the systemic circulation at the superior vena cava. We can then make various hypotheses concerning the metabolic pathways of the chyle. The simplest would be Hypothesis 1 illustrated in Fig. 2.

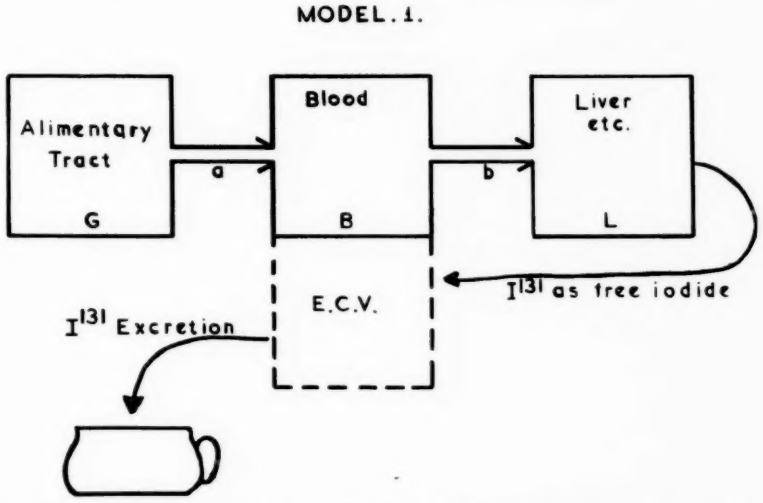


Fig. 2.—*Model 1.* The "single channel" model of lipid metabolism, in which the absorbed fat all follows the same metabolic route. In this and all subsequent diagrams, a double line indicates the passage of lipid material in which the I^{131} marker is firmly bound. A single line indicates the movement of the I^{131} liberated as a result of oxidation of the lipid.

Hypothesis 1.—The fat is absorbed from the small intestine at a given rate, a, into the blood via the lymphatics. From the blood it is cleared to the liver and fat depots at a rate, b. The liver oxidizes the fat at a rate, c, and during this process the iodide is detached from the oleic acid chain from which it diffuses through the iodide space and is finally excreted by the kidneys.

On this hypothesis, the wave of bound radioactivity (Fig. 1, *B*) is to be understood in terms of a competition between the absorption rate, a, and the clearance rate, b. A similar situation arises in the glucose tolerance curve.

It is quite easy to prove that Hypothesis 1 is false. This has been done mathematically but the equations need not be repeated here. The incompatibility arises between the slow rate of clearance (half-time about 9 hours) and the low level, approximately 5 per cent, of the administered dose, attained by the maximum in the curve of the labeled fat as seen in Fig. 1, *b*. It is impossible for such a slow clearance rate to be a decisive factor in holding the serum concentration to such a low level as 5 per cent. We are, therefore, forced to conclude that there are two forms of lipid in the postalimentary lipemia, and we consider as a tentative working hypothesis, the following:

Hypothesis 2.—Human chyle contains two fractions which we shall refer to as α- and β-fractions. The α-fraction contains about 5 per cent of the ingested material. It is cleared slowly by the liver with a half-clearance time of 9 hours ($b \sim 0.075$ hour^{-1}). It is responsible for most of the postalimentary lipemia.

The β-fraction contains about 95 per cent of the ingested lipid. It is cleared in negligible time from the blood by the fat depots. From the depots, this component may be quickly transferred to the liver. Both α and β fractions are oxidized in the liver. In the oxidation process the I^{131} marker is liberated and returns to the extracellular volume, from which it is excreted in one or two days.

Models which conform to these ideas are shown in Fig. 3, and we will now set down and solve the equations demanded by this hypothesis. The equations describe each of the physically different models *2a*, *2b*, and *2c*. Since the two fractions follow out their own pathways, we shall consider their mathematical formulation separately, adding together the effects of each channel where applicable.

Theory of α-fraction

Let G = total labeled lipid in the alimentary tract; B_1 = amount of bound radioactivity in the systemic circulation; B_2 = amount of free iodide arising from metabolism of B_1; L = total of labeled lipid in the liver and other depots; U_1 = total radioactivity excreted in the urine from this fraction; t = time from ingestion of test meal; a = rate of absorption from alimentary tract; b = rate of fixation in depots; c = rate of metabolic turnover; and k = rate of urinary excretion.

The following are the differential equations describing the flow of radioactivity between the various compartments of Fig. 2.

$$\frac{dG}{dt} = -aG \tag{3}$$

$$\frac{dB_1}{dt} = \alpha aG - bB_1 \tag{4}$$

$$\frac{dB_2}{dt} = cL - kB_2 \tag{5}$$

$$\frac{dL}{dt} = bB_1 - cL \tag{6}$$

$$\frac{dU_1}{dt} = kB_2 \tag{7}$$

These are the normal equations one meets in the description of chemical or biologic processes.

Equation (3) expresses the fact that the amount of fat absorbed per unit time through the intestinal mucosa is proportional to the amount of fat present in the lumen.

Equation (4) states that the amount of fat appearing in the circulation per unit time is equal to the amount of fat absorbed multiplied by the partition coefficient, less the amount of fat deposited in the depots (bB_1).

Equations (5) and (6) are similar, but equation (7) merely says that the amount of iodine excreted per unit time is proportional to the total amount in the iodide space.

We have the following conditions:

$$\left.\begin{aligned} \text{At } t = 0,\ G &= G_0 \\ B_1 = B_2 = L = U &= 0 \end{aligned}\right\} \tag{8}$$

and at all times

$$\alpha G + B_1 + B_2 + L + U = \alpha G_0 \tag{9}$$

The solutions of equations (3) to (7) with the given conditions are:

$$G = G_0 e^{-at} \tag{10}$$

$$B_1 = \frac{\alpha a G_0}{b - a} (e^{-at} - e^{-bt}) \tag{11}$$

$$B_2 = \frac{\alpha abe G_0}{(b - a)(k - e)} \left\{ B_2' e^{-at} + B_2'' e^{-bt} + B_2''' e^{-et} + B_2'''' e^{-kt} \right\} \tag{12}$$

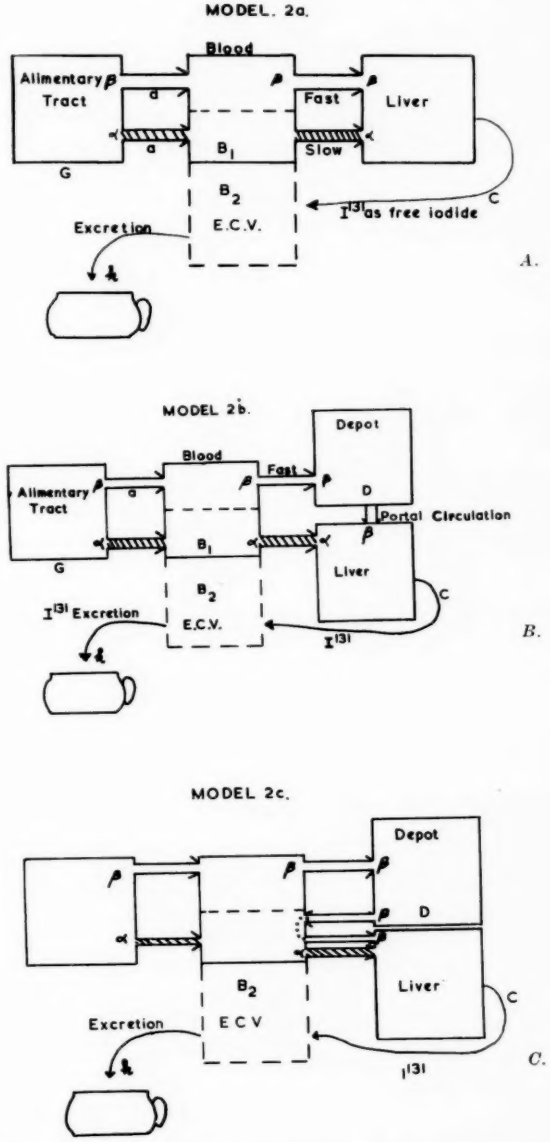


Fig. 3.—*Model 2.* Various possible versions of the "two channel" model of lipid metabolism. The absorbed fat appears in the blood stream in two separate fractions, α and β, which are cleared slowly and quickly, respectively. These models differ only in the physical pathways of the β-fraction, and are the same mathematically. *A*, The β-fraction moves quickly to the liver; *B*, and *C*, it moves to the fat depots and from these to the liver either via the portal circulation *(B)* or the systemic circulation *(C)*.

where

$$\left.\begin{aligned} B_2' &= \frac{1}{e-a} - \frac{1}{k-a} \\ B_2'' &= \frac{1}{k-b} - \frac{1}{e-b} \\ B_2''' &= \frac{1}{e-b} - \frac{1}{e-a} \\ B_2'''' &= \frac{1}{k-a} - \frac{1}{k-b} \end{aligned}\right\} \tag{13}$$

$$L = \frac{\alpha a b G_0}{b-a}\left\{\frac{(e^{-at}-e^{-ct})}{e-a} - \frac{(e^{-bt}-e^{-ct})}{e-b}\right\} \tag{14}$$

$$U_1 = k\int_0^t B_2\,dt = \frac{k\alpha a b c G_0}{(b-a)(k-c)}\left\{\begin{aligned} &B_2'\frac{1-e^{-at}}{a} + B_2''\frac{1-e^{bt}}{b} \\ &+ B_2'''\frac{1-e^{-ct}}{c} + B_2''''\frac{1-e^{-kt}}{k}\end{aligned}\right\} \tag{15}$$

Theory of β-fraction

The appearance of the lipid of this fraction in the blood is so transitory, that we may, without significant error, treat the tracer as if it goes straight from the alimentary tract to the liver or depots.

Let L = amount of labeled lipid in liver or depot; B = amount of free iodide in extracellular volume; U_2 = total radioactivity excreted from this route.

In terms of the same constants, a, c, and k, defined previously, the differential equations for this route are:

$$\frac{dG}{dt} = -aG \tag{16}$$

$$\frac{dL}{dt} = \beta aG - cL \tag{17}$$

$$\frac{dB}{dt} = cL - kB \tag{18}$$

$$\frac{dU}{dt} = kB \tag{19}$$

Equation (18), for example, says that the rate of increase in the free iodide is given by the amount coming from the liver in unit time (cL) minus the amount excreted in unit time (kB).

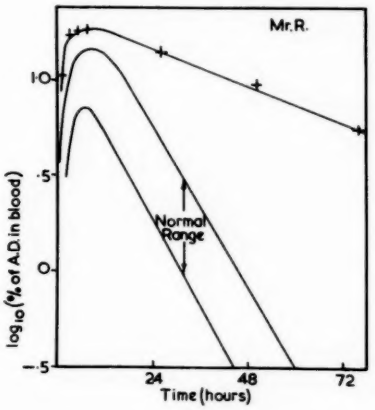
The solutions for the usual boundary conditions are:

$$G = G_0 e^{-at} \tag{20}$$

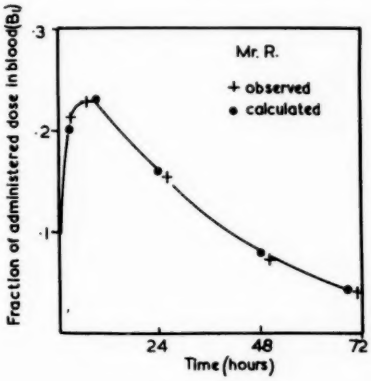
$$L = \frac{\beta a G_0}{c-a}(e^{-at} - e^{-ct}) \tag{21}$$

$$B = \frac{\beta a c G_0}{c-a}\left\{\frac{e^{-kt}-e^{-at}}{a-k} - \frac{e^{-ct}-e^{-kt}}{k-c}\right\} \tag{22}$$

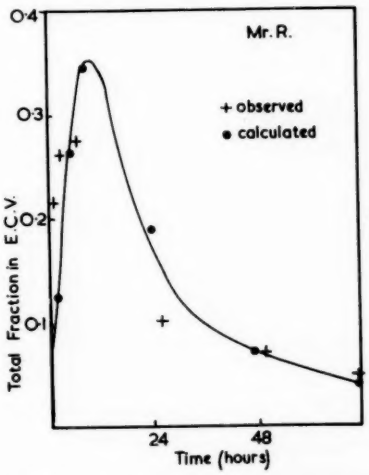
$$U = \frac{\beta G_0}{c-a}\left\{\frac{ca(1-e^{-kt}) - kc(1-e^{-at})}{a-k} + \frac{ca(1-k^{-kt}) - ka(1-e^{-ct})}{k-c}\right\} \tag{23}$$



A.



B.



C.

Fig. 4.—*A*, *B*, and *C*, The total radioactivity and bound and free radioactivity are given separately in a case of hyperlipemia and xanthomatosis. In these diagrams are also given values calculated on the basis of Model 2.

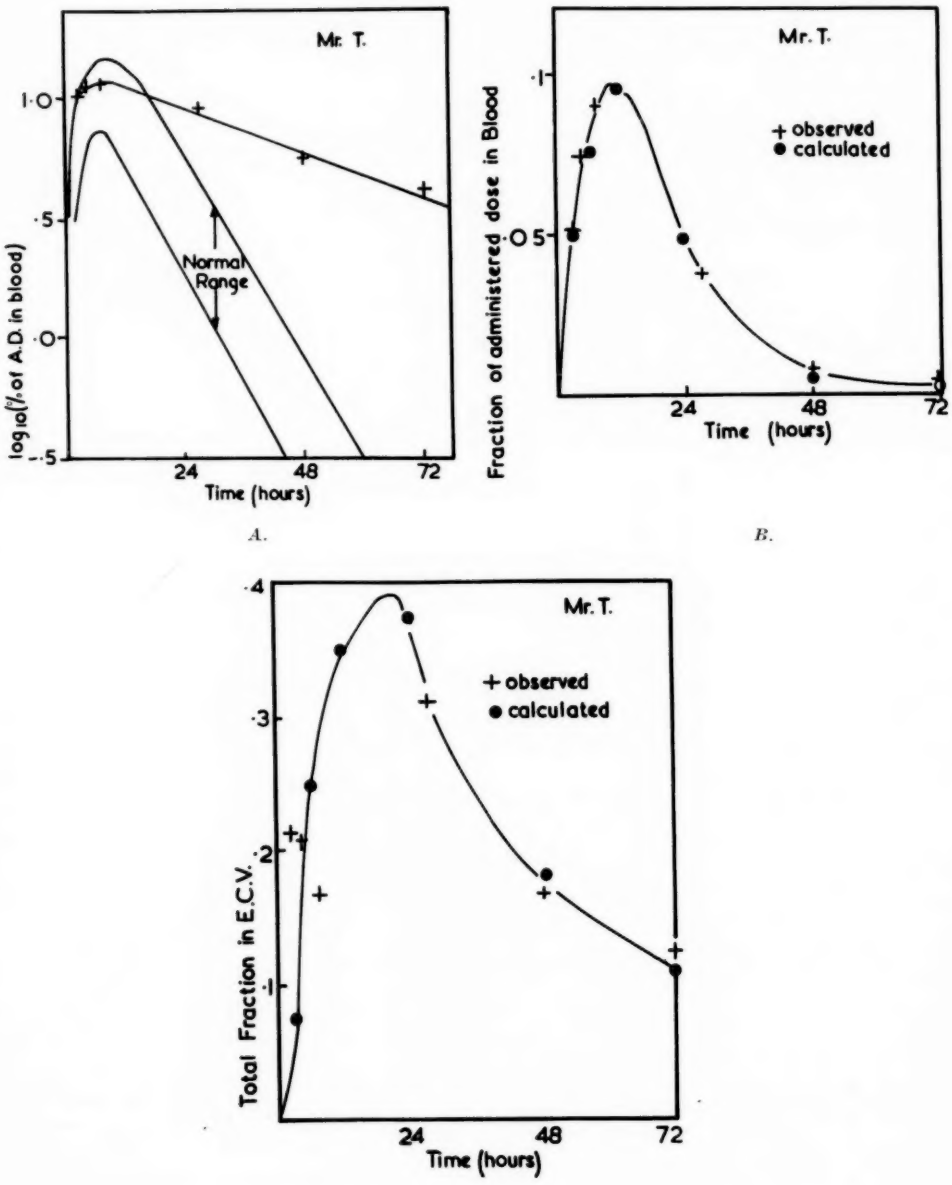


Fig. 5.—Total, bound, and free radioactivity in a case of myocardial infarct. Values calculated from Model 2 are also indicated.

Expanding U in powers of t, it is found that the first term different from zero is the term in t^3, and that for t small,

$$U = \frac{\beta G_0 cakt^3}{6} \qquad (24)$$

The total of free iodide in the extracellular volume and the total urine excreted will be given by the sum of the components arising from each route, i.e., from (12) and (22):

total free iodide/G_0

$$= \frac{\alpha abc}{(b-a)(k-c)}\left\{B_2'e^{-at} + B_2''e^{-bt} + B_2'''e^{-ct} + B_2''''e^{-kt}\right\}$$
$$+ \frac{\beta ac}{c-a}\left\{\frac{e^{-kt}-e^{-at}}{a-k} - \frac{e^{-ct}-e^{-kt}}{k-c}\right\} \qquad (25)$$

where B_2', etc., are given in equation (13). Similarly from (15) and (23):

total urinary excretion/G_0

$$= \frac{\alpha abck}{(b-a)(k-c)}\left\{B_2'\frac{1-e^{-at}}{a} + B_2''\frac{1-e^{-bt}}{b} + B_2'''\frac{1-e^{-ct}}{c} + B_2''''\frac{1-e^{-kt}}{k}\right\}$$
$$+ \frac{\beta}{c-a}\left\{\frac{ca(1-e^{-kt}) - kc(1-e^{-at})}{a-k} + \frac{ca(1-e^{-kt}) - ka(1-e^{-ct})}{k-c}\right\} \qquad (26)$$

COMPARISON WITH EXPERIMENTAL RESULTS

Normal Values.—So far this approach has been applied to the analysis of some 40 lipid tolerance curves. Good agreement between calculated and observed results was always obtained.

Twenty of the tests were made on normal controls, and from the results in this group the normal ranges for the various rate constants, α, a, b, etc., have been determined. The results are given in Table I.

TABLE I

CONSTANT	NORMAL RANGE
Value of α-fraction	0.03 to 0.12
Absorption rate, a	0.2 to 1.3
Clearance rate, b	0.05 to 0.12
Oxidation rate, c	0.1 to 0.3
Renal clearance rate, k	> 0.06

A typical set of normal curves is shown in Fig. 1. Also shown in these diagrams are the values calculated for $\alpha = 0.045$, a = 0.31 hour^{-1}, b = 0.107 hour^{-1}, c = 0.2 hour^{-1}, and k = 0.15 hour^{-1}.

Abnormal Curves.—The same agreement also always can be obtained in cases of disordered lipid metabolism; this will be illustrated in one or two cases.

1. Patient R., a 52-year-old man, had familial hyperlipemia and xanthomatosis. The curves obtained in this case are shown in Fig. 4. The following choice of constants was found to fit the curves: $\alpha = 0.295$, raised; a (absorption) = 0.44, normal; b (clearance) = 0.03, low; c (oxidation) = 0.2, normal; and k (renal) = 0.15, normal.

Values calculated using these numerical constants are also shown in Fig. 4. The combination of a raised α-fraction and reduced clearance rate both serve to give rise to a high serum lipid level and to maintain it for a significantly long time. These results would be in keeping with the clinical findings.

2. Patient T., a 50-year-old man, had coronary occlusion and acute pulmonary edema. The experimental results are shown in Fig. 5, as are those calculated for the following constants: $\alpha = 0.25$, raised; a (absorption) = 0.09, low; b (clearance) = 0.11, normal; c (oxidation) = 0.4, normal; and k (renal) = 0.06, normal.

The existence of a raised value of the α-fraction is a typical finding in patients with coronary disease.

3. Patient Z., a 71-year-old woman, had myxedema. The results are shown in Fig. 6. The following constants were found: $\alpha = 0.052$, normal; a (absorption) = 0.385, normal; b (clearance) = 0.031, low; c (oxidation) = 0.06, low; and k (renal) = 0.08, normal.

In this case the patient's plasma clearance rate and oxidation rates were found to be reduced and this is presumably consistent with the established diagnosis of myxedema.

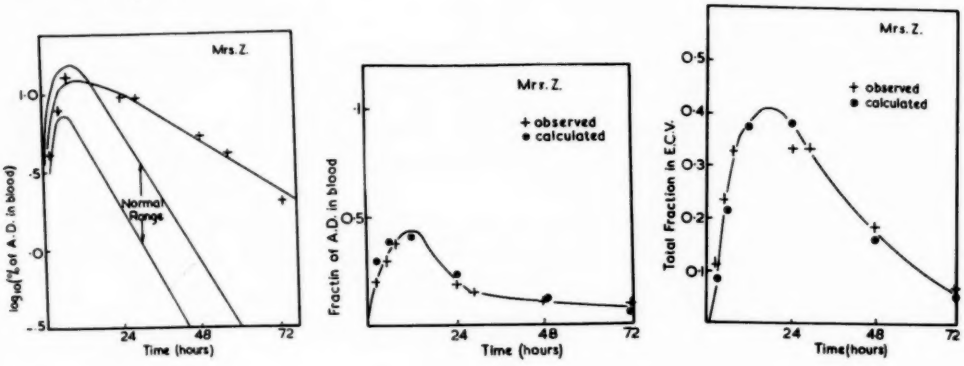


Fig. 6.—Total, bound, and free radioactivity in a case of myxedema, and comparison with values calculated from Model 2.

DISCUSSION

It has been shown that the single channel theory of Hypothesis 1 does not agree with observations of the metabolism of triolein in man. Also, it has been shown that the two channel theory of Hypothesis 2 does agree with those observations.

On the other hand, the various physically different models illustrated in Fig. 3, *A*, *B*, and *C* are consistent with Hypothesis 2, and further observations are necessary to distinguish between them. Some attempts to distinguish between these models have been made by making external counting measurements at the body surface of suitable patients. In these patients the labeled material was clearly traced from the stomach into the small bowel in periods of a few hours. These results will be more fully described elsewhere. The important thing for our present purpose is that at no time was there a significant concentration of the isotopically labeled material in the liver. Thus, we can conclude that Model 2a is not consistent with observation and that Models 2b and 2c are still possible.

In these models, the β-fraction is rapidly cleared into the fat depots. From there it is transferred to the liver where it is oxidized. This transfer may take place via the portal circulation (Model 2b) or the systemic circulation (Model 2c) or possibly both. Experiments are planned in which measurements of the level of radioactivity in the portal blood will be made after the ingestion of isotopically labeled lipid. It is hoped in this way to throw some light on the validity of Models 2b and 2c, but this has not yet been done.

In equation (3) we have assumed that the amount of fat absorbed per unit time is proportional to the amount of fat in the alimentary canal. It has been suggested to us[4] that it might be preferable to assume that the lipid is removed from the alimentary canal in a linear rather than an exponential manner. Thus, one might assume that the absorption process occupies a certain time, T hours, and that the absorption rate is constant at the rate G_0/T. On this model, the absorption rate is specified by the value of T, and 1/T plays approximately the same role as a in our model. Substituting this alternative assumption into the equations makes surprisingly little difference to the calculated figures. This is because in the majority of cases the absorption process is not one of the dominating factors in the lipid metabolic process. In a series of normal subjects, we find values of T ranging from 1 to 5 hours, corresponding to the normal range found for values of the absorption constant a of 0.2 to 1.3. Even with this modified approach to the low adsorption, this leaves unmodified our main conclusion, namely, that the chyle contains two main fractions comprised in the proportions of 5 and 95 per cent approximately, and with very different removal rates. As to the nature of these two fractions, one can at this stage only speculate. Work is in hand at the present moment in order to identify, by biochemical methods, the nature of these two fractions.

It is of interest to note that French and Morris[5] made intravenous injections of homologous chyle taken from other rats in the postprandial state. These workers used C^{14}-labeled tripalmitin and showed that the removal of this chyle from the circulation was a two component curve. The rapidly removed component comprised 95 per cent of the chyle, and the time for half-clearance of this fraction was of the order of 5 to 10 minutes. The remainder, comprising 5 per cent of the chyle, was cleared with a half-time of 65 minutes. These findings are very similar to our findings in man, with the exception that we find in man that the half-time of the slowly cleared fraction is closer to 9 hours.

In discussing the removal of lipids from the blood stream, French and associates[5] showed that in the postprandial state the lipids in man may be separated into three main fractions: (1) lipoproteins, (2) chylomicrons, and (3) unesterified fatty acids. Bergström and Borgström[6] showed that most of the postprandial lipid was in the form of chylomicrons, and Havel and Fredrickson[7] and French and Morris[8] showed that the circulating half-life of this fraction was 5 to 10 minutes. For the lipoproteins the circulating half-life was found by Zilversmit and Bollman[9] to be 1 hour in the rat, and by Hahn and Hevesy[10] to be 1½ hours in the rabbit. Zilversmit and collaborators[11] found this figure to be 5 to 8 hours in the dog. Thus the rate of removal of the lipoprotein fraction is obviously species dependent, and a value of 9 hours would be quite admissible in man. The unesterified fatty acids form only a small fraction of the chyle[6] and have a circulation half-life in man of only 2 minutes.[12] Hence, they do not play a significant role in our observations. In the light of the foregoing, it would be tempting to equate our so-called α-fraction with the lipoprotein moiety, and our β-fraction with the chylomicrons. However, this is pure speculation and must await the results of the biochemical investigation at present in hand.

In the course of this investigation, several other models were considered and worked out mathematically. None of them was found to be satisfactory.

Model 3 is illustrated in Fig. 7. The fat was considered to be all of one type which was cleared fast to the liver. Here most of it (the β-fraction) was oxidized while the α-fraction was recirculated into the systemic blood and finally cleared by the liver. This model was discarded in the light of our experimental finding of an absence of a significant concentration of labeled ingested triolein in the liver.

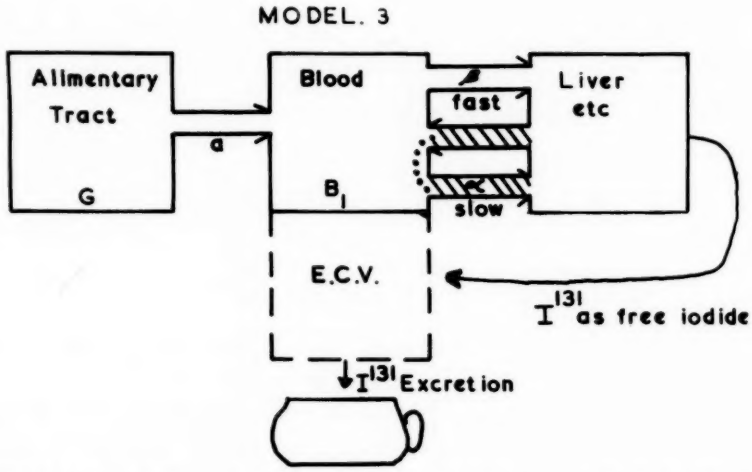


Fig. 7.—*Model 3,* One of the models found to be unsatisfactory as a basis for the metabolism of triolein in man.

Another possible model, Model 4, is shown in Fig. 8. Here, all the fat in the blood is of the same type and is not separated into two distinct channels. From the blood, the fat is cleared into depots and liver with different rate constants p and q. (We know $p > q$.) The depot fat then returns to the circulation with rate constant m. After which some goes to the liver but most goes again to the depots since $p > q$.

The labeled fat is thus continually recycling between systemic blood and depots. This is absurd and the rate of the rise of iodide in the blood would be much too small and too delayed.

Model 5, shown in Fig. 9, is a variant of Model 3 modified to overcome the observed absence of liver counts. This model was also found to be in disagreement with observed results.

Thus, of the seven different models of lipid metabolism which have been considered, only two give equations in satisfactory agreement with the observed lipid tolerance curves. These are the Models 2b and 2c shown in Fig. 2. These models have in common the requirements that human chyle contains two main fractions referred to as the α- and β-fractions. The numerical values of α and β together with the other constants determine the metabolic development of these two fractions and form a sufficient basis for understanding the

handling of postprandial lipemia in man. The departure of one or more of these constants from the normal in patients with disordered lipid metabolism may then serve as a useful guide as to the nature, site, and degree of lipid metabolic disorder.

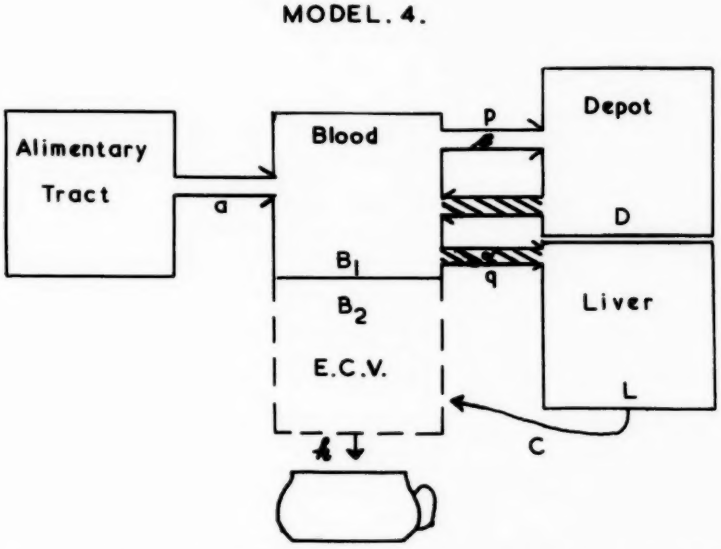


Fig. 8.—*Model 4*, Also unsatisfactory as a model for the metabolism of triolein in man.

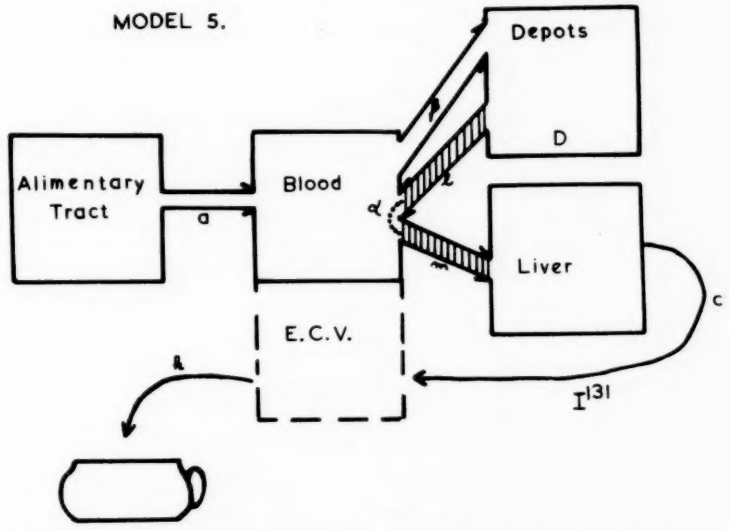


Fig. 9.—*Model 5*, An unsatisfactory model for the metabolism of triolein in man.

CONCLUSIONS

From the work described in this article, we have come to the following conclusions concerning the metabolism of triolein in man.

1. The single channel type of theory, in which the ingested lipid follows the same metabolic pathway, disagrees by a large factor with observed results.

2. Double channel type of theories do agree with observation. In this type of theory, human chyle, absorbed from the alimentary canal at a rate, a, is thought to comprise two separate fractions: an α-fraction comprising about 5 per cent and a β-fraction comprising about 95 per cent of the chyle. The α-fraction is cleared slowly ($\sim$ 9 hours) whereas the β-fraction is cleared rapidly (about 5 to 10 minutes).

3. The observations are consistent with the assumption that these lipid fractions are oxidized at a certain rate, c, and in the process of oxidation the I^{131} marker on the oleic acid is liberated and moves in the iodide space where it is excreted via the renal route with a rate constant k.

4. These various processes may be described by certain rate constants which have the following ranges in normal man (units: $hour^{-1}$): a, the rate of absorption, 0.2 to 1.3; b, the rate of clearance of the α-fraction 0.05 to 0.12; c, the rate of oxidation 0.1 to 0.3; and k, the rate of renal clearance greater than 0.06.

5. The evaluation of these various constants in patients with disordered lipid metabolism may serve as a useful guide in the evaluation of the cause and degree of disorder.

6. Further observations are needed to identify the nature of the α and β components.

7. Various physical alternatives of the double channel type of theory are illustrated. Of these, one model is found to be inconsistent with observation. We are not at this stage able to distinguish between the other two models.

Addendum.—Further observations have weakened the argument against Model 3 (Fig. 7) and, therefore, this must be admitted as a possible version of the two-channel type of model.

We are grateful to the Sisters of Charity for providing facilities for this investigation. We would like to thank Professors C. R. Blackburn and Bengt Borgström for helpful discussions. Drs. Malcolme Whyte and Warwick Browne helped us clarify many of our ideas. One of us (G. S. F.) wishes to acknowledge the award of the Hungarian Fellowship in Nuclear Medicine, and more recently, financial assistance from the Post-graduate Medical Foundation, University of Sydney.

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THE CELL POPULATION OF HUMAN LIVER

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In six surgical biopsies of normal human liver, the average number of nuclei was (223 ± 13) times 10^3 per milligram wet weight of liver. The distribution of parenchymatous and littoral cells was determined in 23 punch biopsies from normal human liver. Allowing for the presence of 10.9 per cent of binucleate parenchymatous cells, the total number of cells per milligram of human liver is 202 times 10^3 and, of these, approximately 171,000 are parenchymatous and 31,000 are littoral cells.

RECENT attempts to base biochemical determinations on the parenchymatous liver cell in the rat[1, 2] have been helped by a knowledge of the cellular composition of rat liver.[3] We have studied the cellular composition of human liver.

MATERIALS AND METHODS

Twenty-three punch biopsy specimens were obtained from patients at the Stadtkrankenhaus Kassel. None of these had any clinical evidence of liver disease. Examination of the serum in these patients revealed normal concentrations of bilirubin, albumin, and globulin. Flocculation tests and sulfobromophthalein retention were likewise within normal limits. The liver appeared normal grossly to peritoneoscopic vision and microscopically to two independent pathologists. A portion of the tissue removed was assayed for protein content and for between 6 and 17 enzymes of intermediary metabolism and found to be within the normal limits previously determined.[4] In general, these procedures were carried out at the request of the referring physician. Four paraffin sections, 5 μ in thickness, were cut from each biopsy specimen and stained with hematoxylin and eosin. In each section, the distribution of 1,000 consecutive nuclei between parenchymatous, littoral (sinusoidal lining cells including Kupffer cells), bile duct, blood vessel, and connective tissue cells were determined. A total of 92,000 nuclei were, therefore, examined.

In each of the 23 specimens, the nuclear lengths of 50 parenchymatous and littoral cells were measured under oil immersion. In the case of the nonspherical nuclei of the littoral cells, the largest diameter was measured. This permitted the apparent distribution obtained from direct counts to be adjusted for differences in nuclear size to give the true distribution of nuclei whose center points lay within the section being counted.[5]

Total nuclei counts were made on homogenates from wedge biopsies obtained from six patients at laparotomy performed for nonmalignant conditions of the abdomen not involving the biliary tree. The biopsy specimen was chilled, the capsule excised, and the tissue weight determined immediately. In view of the observation that prolonged homogenization results in nuclear disintegration,[6] the conditions for obtaining maximum yield of nuclei were standardized on fresh autopsy material. A Potter Elvehjem glass homogenizer

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with a longitudinally grooved Teflon pestle 2 cm. in diameter was used throughout. Homogenization in 0.85 per cent saline gave nuclear yields which were as high as or higher than those obtained by the use of 0.25M sucrose, the medium used traditionally for the preservation of particulate cellular material. Saline homogenates showed good and even dispersion of nuclei in the counting chamber. Homogenization for periods of 2, 4, and 5 minutes produced nuclear yields which averaged between 90 and 100 per cent of the value obtained after 3 minutes' homogenization. Homogenization for one minute failed to achieve complete destruction of the cells and a consequent lower yield of nuclei (66 per cent of the 3 minute value). Homogenization for 10 minutes resulted in some loss of nuclei presumably from fragmentation (76 per cent of the 3 minute value). After homogenization the solution was transferred quantitatively to a 25.0 ml. volumetric flask and made to volume with chilled saline. After thorough mixing a 2.0 ml. aliquot was added to 2.0 ml. of hematoxylin in a stoppered polyethylene tube. Two such tubes were prepared from each specimen and nuclei counts were performed in duplicate on a sample from each tube. Although less than 2.5 per cent of nuclei were lost on standing for a period of one hour, all these procedures were carried out rapidly and consecutively. All solutions were kept at 0° C. throughout.

TABLE I. DISTRIBUTION OF CELL TYPES (PUNCTURE BIOPSIES, GERMANY)

CELL TYPE	APPARENT DISTRIBUTION (% ± S. E.)	NUCLEAR LENGTHS (% ± S. E.)	TRUE DISTRIBUTION (%)
Parenchymatous	84.2 ± 2.7	7.12 ± 0.024	86.0
Littoral	14.7 ± 2.2	8.08 ± 0.046	14.0
Bile duct	0.6		
Connective tissue	0.2		
Blood vessel	0.3		

RESULTS

The distribution of cell types in the biopsy material obtained from Kassel is given in Table I. Neglecting the small contribution of bile duct, connective tissue, and blood vessels, 86 per cent of the nuclei of the human liver are in parenchymatous cells. The mean total nuclei count on the surgical biopsy specimens was (223 ± 13) times 10^3 cells per milligram wet weight. The cellular distribution of 4 of the 6 surgical biopsy specimens was determined from stained sections and found to be comparable with that of the material obtained from Kassel (Table II). As 10.9 per cent of the parenchymatous cells were found to be binucleate, the average number of cells per milligram of human liver is 202 times 10^3 and of these, 171,000 are parenchymatous cells and 31,000 are littoral cells.

TABLE II. DISTRIBUTION OF CELL TYPES (SURGICAL BIOPSIES), MICHIGAN

SPECIMEN NO.	DISTRIBUTION (%)			BINUCLEATE PARENCHYMATOUS CELLS (%)
	PARENCHYMATOUS	LITTORAL	OTHERS	
1	79.7	18.7	1.6	7.2
2	82.5	12.7	4.8	9.7
3	80.7	17.0	2.3	5.0
4	76.3	17.8	5.9	8.9
Mean	79.8	16.6	3.6	7.8

SUMMARY

The distribution of cell types from normal human liver was determined by microscopic examination of 23 punch biopsy specimens and nuclei counts on six

normal specimens removed at laparotomy. Normal human liver contains on an average 171,000 parenchymatous cells and 31,000 littoral cells per milligram.

Our thanks are due Prof. W. Welpler and Dr. Murray R. Abell for examining our slides and to Dr. C. Gardiner Child, III, and the members of the Department of Surgery, The University of Michigan, who kindly provided the liver biopsy specimens. We are especially grateful to Dr. Abell for his continuous help and advice in the course of this work.

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THE PROPERDIN SYSTEM AND IMMUNITY

XV. Some Biologic Effects of the Administration of Zymosan and Other Polysaccharides to Rabbits, and the Presence of Antibodies to Zymosan in Human and Rabbit Serum

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Properdin interacts in vitro with zymosan and other high molecular weight polysaccharides. It has been suggested that properdin is a nonspecific serum factor in mechanisms of natural defense. An alternative proposal suggests that properdin is an antibody to zymosan with broad cross reactivity.

The present studies were initiated to investigate the biologic effects of zymosan in vivo in rabbits, and to study the properties of antibodies to zymosan developed in the serum of injected animals and occurring naturally in humans. Parenteral administration to rabbits of zymosan, native dextrans, and native levans which interacted with properdin in vitro caused diminution in serum properdin and C'3 levels. Although the agents were pyrogenic, properties responsible for interacting with properdin and C'3 differed from endotoxic properties. The agents active in vitro and in vivo also were capable of eliciting the local Shwartzman reaction after skin preparation with endotoxin.

Agglutinating properties for zymosan appeared in the immunized animals. These had different distribution, properties, and requirements from properdin. By appropriate studies they and the agglutinins for zymosan which occur in normal human serum were distinguishable from properdin. Thus, properdin is considered distinct from the antibodies to zymosan.

ZYMOSAN, the insoluble residue of yeast, composed primarily of carbohydrate, interacts in vitro with human serum to remove a protein, properdin, from serum and to inactivate the third component of serum complement, C'3.[1] A variety of high molecular weight polysaccharides of bacterial origin, including native levans and dextrans, and lipopolysaccharides from gram-negative bacteria, also interact with properdin in vitro.[2] The present study was undertaken to investigate the effects of zymosan and other high molecular weight polysaccharides in rabbits. Observations were made on alterations of levels of properdin and serum complement, production of fever, alteration of leukocyte counts, development of the Shwartzman phenomenon, the production of antibodies to zymosan, and the effect of such antibodies in rabbit and human serum

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†Deceased.

on the properdin system. The results indicate that the ability of polysaccharides to alter properdin levels after injection in vivo correlates with in vitro properties for interaction with the properdin system. They also offer evidence that properdin is distinct from the agglutinins for zymosan found in serum.

The nomenclature is that used previously in the series of articles from these laboratories.[3]

MATERIAL

Animals.—Albino rabbits of mixed stock and mixed sexes weighing 2,500 to 3,500 grams were used, except for studies on the generalized Shwartzman reaction in which animals weighing 1,000 to 1,200 grams were used. No rabbit was used for more than one experiment. During studies with pyrogen the animals were housed in a temperature controlled room at 25° C. and confined to a small box when blood samples were obtained or temperature was determined. Temperatures were determined rectally with a clinical thermometer; multiple control determinations were performed to assure stabilization and tolerance to handling. Temperatures were recorded at 15 to 30 minute intervals for the first hour after injection and every 60 minutes thereafter.

Materials Injected.—All material injected was suspended or dissolved in sterile, nonpyrogenic 0.15M sodium chloride to a total volume of less than 10 ml. Glassware was cleaned with detergent and acid, and rinsed multiple times in tap and distilled water. No special treatment was used to render glassware pyrogen-free. However, when this glassware was used for injections of saline no fever resulted.

Zymosan* was prepared for injection as described previously as a suspension of the insoluble material.[3] The preparations used interacted with properdin in vitro.

Native levan No. 4 was supplied by Dr. S. Hestrin, Jerusalem, Israel.† These materials were obtained in lyophilized preparations. They were dissolved in 0.15M sodium chloride in viscous solution. Properties of these materials with respect to the properdin system have been reported previously.[2]

Typhoid-paratyphoid vaccine‡ contained 100 million organisms per milliliter.§

METHODS

All injections and bleedings were made in the contralateral marginal ear veins. Blood samples were obtained by severing the vein, collecting the blood into a chilled tube, and allowing it to clot and retract at 4° C. Samples were centrifuged at 0° C. and stored in a mechanical freezer at −65° C. The samples were handled in the cold to avoid interaction during clotting between serum and the materials injected. Leukocyte counts and differential smears were made from a drop of free flowing blood from the marginal ear vein. During the studies on the Shwartzman reaction, organs for gross and microscopic examination were obtained immediately after death. Surviving animals were killed 24 hours after the final injection.

Levels of serum properdin were determined by the zymosan assay, using reagents selected for measurements in animal serum.[3] The method is reproducible to ± 33 per cent.[14]

*Zymosan was supplied by Fleischman Laboratories, Standard Brands, Inc. (Nos. 5B151 and 5B171).

†The other native levan and the native dextrans were supplied by Dr. Allene Jeannes, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Ill.

‡Typhoid-paratyphoid vaccine was obtained from Mr. A. S. Hammond, Lederle Laboratories, Pearl River, N. Y.

§Other endotoxin preparations used in producing the Shwartzman reaction were kindly supplied as follows: crude meningococcal filtrate, Dr. Richard T. Smith, University of Minnesota, Minneapolis, Minn.; crude endotoxin from *Salmonella typhosa*, Dr. Maurice Landy, National Cancer Institute, Bethesda, Md.; endotoxin from *Escherichia coli*, Mr. Aaron Lane, Difco Laboratories, Detroit, Mich.

Measurements of serum complement and the four components of complement (designated C'1, C'2, C'3, and C'4) were performed by the double dilution technique, with a reproducibility of ± 50 per cent, using reagents made from human serum by methods described elsewhere.[4-7] Rabbit C'3 can be measured in human R_3, which was also used for the determinations on rabbit serum.

The presence of agglutinins to zymosan was determined on a glass tile, by macroscopic observation of clumping of mixtures of equal volumes of test serum and zymosan suspension when viewed over a substage light after agitation for 5 minutes at room temperature. Microscopic examination yielded similar results. For rabbit sera a suspension of 5 mg. per milliliter of zymosan in saline was used. For human sera a zymosan suspension of 1 to 3 mg. per milliliter was finely divided in a ground glass homogenizer to minimize spontaneous clumping due to the larger particles in the usual zymosan suspension.

The effect of antibodies to zymosan on the inactivation of the third component of complement (C'3) in serum was determined by mixing 0.25 ml. of inactivated antiserum with 1 ml. of normal human serum that contained 2 mg. of zymosan, and incubating the mixture at 37° C. for 60 minutes. The separate components of complement were measured after centrifuging to remove zymosan.

RESULTS

Injection into individual rabbits of 2, 10, 20, 40, 60, 100, and 200 mg. of zymosan No. 5B151 per kilogram of body weight consistently caused a marked fall in the serum properdin level (Fig. 1). The decrease was detected in

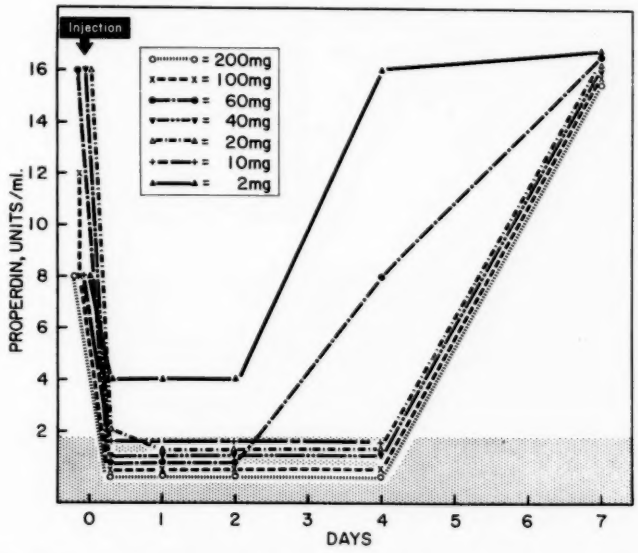

Fig. 1.—Change in serum properdin level of rabbits after intravenous administration of various doses of zymosan. (Limit of assay 2 units. Points in shaded area indicate no activity detected at 2 unit level.)

samples drawn 15 minutes after injection, and at all doses greater than 2 mg. per kilogram no properdin could be detected in the serum for 2 to 4 days thereafter. It should be emphasized that only single animals received each dose of zymosan, and individual variations in the "rebound" pattern may account for some of the differences observed.

Properdin titers were maintained at low levels by repeated injections of 10 mg. per kilogram of zymosan every 1 to 3 days. A series of 12 injections was well tolerated. One animal received daily injections of 60 mg. per kilogram of zymosan and died suddenly after the sixth injection. Examination revealed marked pulmonary atelectasis and occlusion of many pulmonary vessels by material with the same microscopic appearance as zymosan.

Injection of isotonic saline in similar volume did not cause a reduction in serum properdin level. One hundred million killed typhoid and paratyphoid organisms and lethal and sublethal amounts of endotoxin derived from typhoid bacilli, meningococci, and coliform organisms caused a marked pyrogenic response but no significant fall in properdin level. Daily injections of 100 million killed typhoid and paratyphoid bacilli for 12 days did not result in alteration of the properdin level before or during the development of tolerance to the pyrogenic effects of the killed organisms.

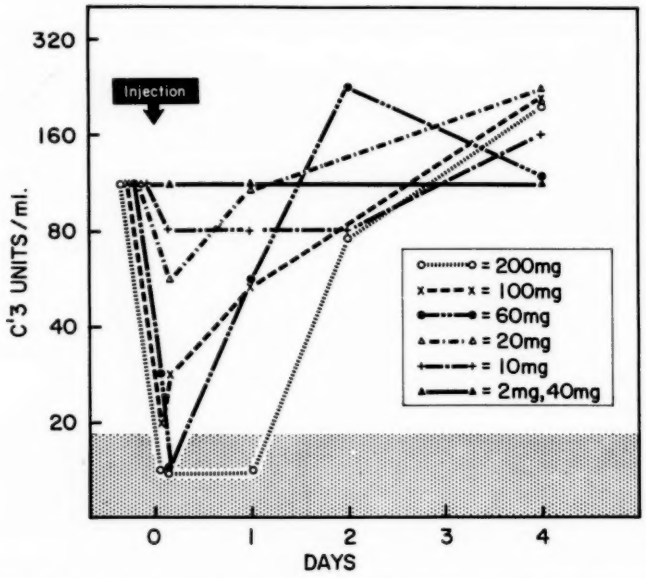

Fig. 2.—Change in serum level of the third component of complement (C'3) after intravenous injection of various doses of zymosan. (Limit of assay 20 units. Points in shaded area indicate that no activity was detected at 20 unit level.)

Complement.—The intravenous injection of zymosan into the same rabbits resulted in a decrease in the level of the third component of complement (C'3) similar to that which occurs when zymosan is incubated with serum in vitro (Fig. 2). A decrease in C'3 occurred in all animals that received more than 10 mg. per kilogram of zymosan, and the magnitude of change was proportional to the dose administered, decreasing in those animals that received the largest doses to less than 15 per cent of the original value. The C'3 level remained low for one to two days and was followed consistently by a transient rise to values slightly greater than were observed during the control period. Although

the rise in any single animal was of border-line significance, all animals exhibited a similar change. Thus greater amounts of zymosan were required to cause a marked decrease in C'3 than caused a fall in properdin. The other components of complement were not measured.

Pyrogenic Response to Zymosan.—A pyrogenic response which had many characteristics of the pyrogenic reaction after administration of bacterial endotoxin followed the injection of zymosan, as indicated in Fig. 3 which compares the response in two animals that were receiving zymosan and one that was receiving killed typhoid organisms. However, other animals not shown developed monophasic fever curves after injection of zymosan. The duration of

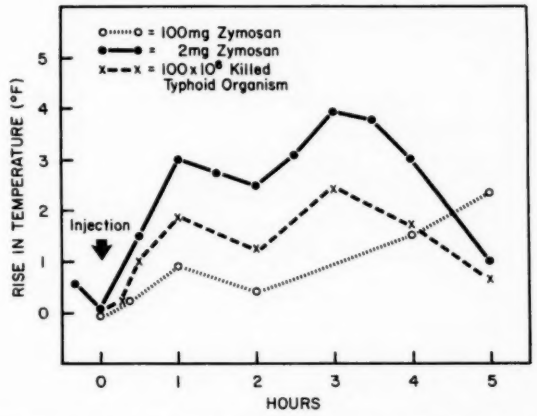

Fig. 3.—Rise in temperature of rabbits after injection of zymosan or bacterial pyrogen.

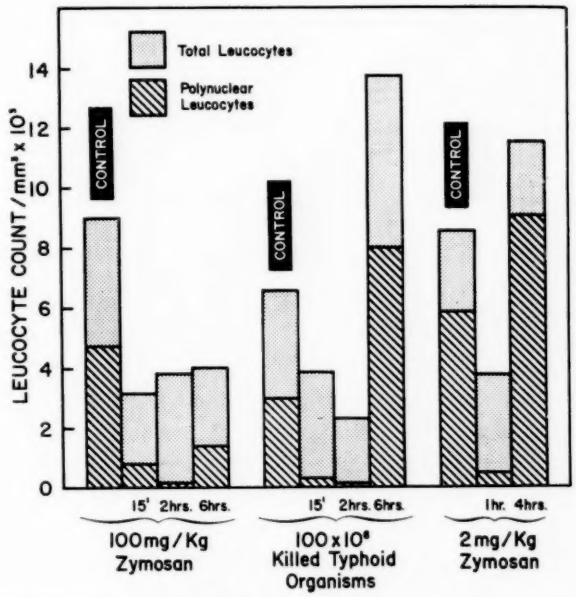

Fig. 4.—Polymorphonuclear leukopenia in rabbits after injection of zymosan or bacterial pyrogen. Injection of the material immediately followed the control determination.

fever was longest with larger doses of zymosan, but the magnitude of the change was variable. There was an immediate decrease in circulating polymorphonuclear leukocytes (Fig. 4) which persisted for longer than the 8 hour period of initial observation when the dose of zymosan was 100 mg. per kilogram or more. During the leukopenic phase the few remaining polymorphonuclear leukocytes often contained granules which had the appearance of zymosan. There was accompanying diarrhea, prostration, and peripheral vasoconstriction similar to that after administration of endotoxin. All the effects were more pronounced with the higher doses, although death did not occur.

The pyrogenic response after daily injection of zymosan progressively diminished in the same manner that the response to repeated doses of endotoxin diminished with the development of tolerance.

Effect of Properdin Level on Response to Pygrogens.—There was no difference in the pyrogenic response to killed typhoid organisms in a pair of animals, one of which had a normal properdin level while the other had an extremely low properdin level (Fig. 5). Similarly, prior administration of zymosan had no effect on the pyrogenic response to killed typhoid organisms.

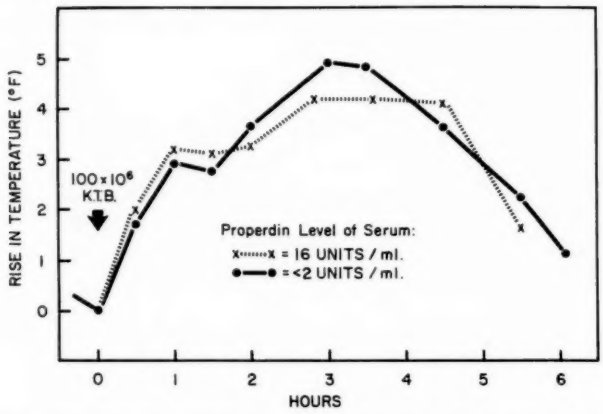

Fig. 5.—Pyrogenic response to bacterial pyrogen of rabbits with low and high serum properdin levels.

Effect of Other High Molecular Weight Polysaccharides on Properdin Level.—Six native dextrans and levans which had been tested in vitro for their ability to interact with properdin were administered to rabbits and serial properdin determinations were performed. Those agents which were active in vitro also resulted in an immediate and marked fall in serum properdin level after administration in vivo (Table I). This occurred within 15 minutes and persisted for variable periods from 7 to 14 days, somewhat longer than occurred after zymosan. The two preparations which were inactive in vitro failed to cause a fall when administered to rabbits in vivo. The ability to cause a fall in properdin did not bear a relation to the pyrogenic properties of the preparations, for equal fever occurred with active and inactive preparations. Variable leukopenia occurred which was not related to the ability to cause a decrease in properdin level.

Properdin System and Shwartzman Phenomenon.—There was no alteration of the serum properdin level during the production of the local Shwartzman reaction by the intradermal injection of bacterial endotoxin followed 18 to 24 hours later by parenteral administration of endotoxin. However, parenteral administration of 10 to 20 mg. per kilogram of zymosan and those dextrans which combined with properdin in vitro and caused a change in properdin level in vivo was effective in precipitating the local Shwartzman phenomenon in rabbits when skin sites had been injected 18 to 24 hours previously with bacterial endotoxin. The two preparations of native dextran which did not combine with properdin failed to elicit the local Shwartzman reaction. However, preparations which were active in eliciting the Shwartzman reaction failed to serve as preparatory agents for the Shwartzman reaction when injected in doses of 10 mg. per kilogram intradermally prior to the parenteral administration of bacterial endotoxin.

The production of bilateral cortical renal necrosis by the intravenous administration of two doses of endotoxin 12 to 24 hours apart, the generalized Shwartzman reaction, was not associated with significant changes in levels of serum properdin. Substitution of zymosan for either dose of endotoxin failed to elicit the generalized Shwartzman reaction, and two doses of zymosan did not produce it. Various agents administered just before or after a single dose of endotoxin have resulted in the development of cortical necrosis.[8] However, administration of zymosan or native dextrans 30 minutes to 2 hours before an intravenous injection of endotoxin, and up to 3 hours after intravenous endotoxin, failed to produce cortical necrosis, although occasional hemorrhagic changes were observed variously in lung, kidney, and intestine. Thus, no direct relation could be demonstrated between properdin level and the local or general Shwartzman reaction, although agents which combine with properdin elicited the local Shwartzman reaction.

Antibodies to Zymosan.—

Antibodies in rabbit serum: The injection of multiple doses of zymosan into rabbits resulted in the appearance of antibodies in the rabbit serum capable of agglutinating various zymosan preparations in vitro. After daily injections of zymosan the antibody appeared approximately 6 days after the initial injection, but did not appear in all animals. It was stable to heating at 56° C. for 30 minutes. Properdin levels at the time of initial appearance of the antibody were usually low because of the repeated doses of zymosan; none were elevated.

The antibody to zymosan did not interfere with the function of zymosan in inactivating C′3, as indicated in Table II. The incubation of zymosan with normal human serum at 37° C. resulted in the complete removal of properdin and inactivation of the third component of complement with little effect on the other components. The addition of rabbit antizymosan serum to fresh normal human serum and zymosan caused agglutination of the zymosan and complement fixation but did not interfere with the inactivation of properdin or C′3. There was no loss of C′1, C′2, or C′4 from the human serum in the presence of antizymosan rabbit serum alone.

TABLE I. RELATION OF IN VITRO AND IN VIVO PROPERTIES OF POLYSACCHARIDES FOR INTERACTION WITH PROPERDIN

PREPARATION	ABILITY TO CAUSE FALL IN PROPERDIN LEVEL	
	IN VITRO	IN VIVO
Native dextran B135582	+	+
Native dextran B1402	+	+
Native dextran B512	0	0
Native dextran B1146	0	0
Native levan B512	+	+
Native levan No. 4	+	+

Antibodies in human serum: Considering the heterogeneity of zymosan,[9] its antigenicity,[10] and the ubiquity of yeasts, it would be expected that antibodies, including agglutinins to zymosan, should be found in human serum. These have been reported by others.[11] Nelson has recently proposed that properdin is a naturally occurring antibody to zymosan.[11] In the present study, agglutinins to zymosan were found in 13 of 25 human sera selected at random. It is to be emphasized that these agglutinins occurred spontaneously, and that none of the individuals were deliberately immunized.

Relation between agglutinin and interaction of serum with zymosan: There was no apparent effect of zymosan agglutinin in human serum on the inactivation of C′3 in serum during incubation with zymosan or on the preparation of R_3 from the serum. On incubation of the 13 human sera that contained spontaneously occurring zymosan agglutinin with 3 mg. per milliliter of zymosan at 37° C. for 60 minutes, C′3 was completely inactivated in all but two. In those sera that showed complete inactivation of C′3, all except two retained concentrations of the other components of complement (C′1, C′2, and C′4) well within the range of normal sera; since they were able to recombine with the other reagents lacking completely one of the other components, they were regarded as making satisfactory R_3 by current criteria.[3, 4] Thus, in contrast to rabbit antizymosan serum the majority of human sera did not cause fixation of the other components of complement. There was no relationship between the presence or absence of demonstrable zymosan agglutinins, the inactivation of C′3, and the residual titer of the other components; the agglutinins did not interfere with the inactivation of C′3.

In only one serum that contained agglutinins and one without agglutinins did a marked fall in C′1, C′2, and C′4 occur during incubation with zymosan, similar to that observed with rabbit antizymosan serum and suggesting that

TABLE II. EFFECT OF RABBIT SERUM ANTIZYMOSAN ON INACTIVATION OF HUMAN COMPLEMENT COMPONENTS AND ON COMBINATION OF HUMAN PROPERDIN WITH ZYMOSAN

HUMAN SERUM	REAGENT (0.25 ML.)	ZYMOSAN 6B14 (MG.)	AGGLUTINATION OF ZYMOSAN	UNITS PER ML. OF SAMPLE AFTER INCUBATION AT 37° C. FOR 1 HOUR				
				C′1	C′2	C′3	C′4	PROPERDIN
1.0	Buffer	2	0	960	160	0	640	< 1
1.0	Normal rabbit serum	2	0	960	160	0	640	< 1
1.0	Antizymosan rabbit serum	2	+	480	40	0	80	< 1
1.0	Antizymosan rabbit serum	—	—	960	160	320	640	8
1.0	Buffer	—	—	960	160	320	960	8

complement fixation occurred. In the serum that contained agglutinins it could be shown that the decrease in C′2 and C′4 during the inactivation of C′3 by zymosan was quite specifically related to the use of zymosan (Table III). Incubation of the serum with a dextran or levan, known to be capable of interacting with properdin and inactivating C′3 in other sera, resulted in complete inactivation of C′3 in this serum, without the marked losses of C′2 or C′4 observed with zymosan. These observations may offer a partial explanation why some sera are not satisfactory for making R_3, which should lack completely C′3, but contain adequate amounts of the other three components of complement.

TABLE III. LEVELS OF PROPERDIN AND COMPLEMENT AFTER INTERACTION OF SERUM AND ZYMOSAN AT 37° C.

SERUM	ADDED REAGENT (2 MG.)	AGGLUTINATION	PROPERDIN	C′1	C′2	C′3	C′4
C. W.	Zymosan	+	< 1	1,920	30	30	1,920
	—		4-6	2,560	480	480	5,120
	Levan 4		< 1	1,920	240	0	3,840
Normal	Zymosan	0	< 1	1,920	240	0	3,840
	—		6	1,920	640	480	5,120

The following data clearly distinguish between properdin and the agglutinins to zymosan found in human serum.

Presence of properdin and zymosan agglutinin in different serum fractions (Table IV): The agglutinin to zymosan was present in Fraction II of serum, which lacks properdin and complement. The agglutinin was present in R1, the serum fraction lacking the first component of complement (C′1) and properdin. The agglutinin was absent from R2, the serum fraction which contains all the properdin, but lacks the second component (C′2). The agglutinin was stable to heating at 56°C. for 30 minutes; properdin, C′1, and C′2 are not.

Removal of properdin from serum without loss of agglutinin: The removal of properdin from serum by absorption with bacterial cell wall stroma (*Shigella dysenteriae,* 17° C. for 60 minutes) did not affect the content of zymosan agglutinin. Both the strength of the reaction and the titer of the agglutination remained unchanged. Similar findings were apparent when properdin (and C′3) was removed from the serum by incubation with bacterial stroma at higher temperatures (37° C. for 60 minutes).

Removal of agglutinin from serum without loss of properdin activity: In every circumstance where agglutination of zymosan was demonstrable in a serum or serum reagent, the agglutinin could be removed from the serum by absorption at 0° C. with zymosan. Generally 5 to 10 mg. of zymosan per milliliter of serum was sufficient to accomplish such removal, although occasionally quantities up to 20 mg. per milliliter were used. R4 and serum to which EDTA was added contain properdin and agglutinin, but lack the necessary components to allow properdin to be adsorbed to zymosan.[1] Thus, under circumstances where properdin could not be removed, the zymosan agglutinin could be removed.

TABLE IV. FACTORS OF PROPERDIN SYSTEM AND AGGLUTININS FOR ZYMOSAN IN VARIOUS SERUM FRACTIONS

REAGENT FROM HUMAN SERUM	FACTORS PRESENT							
	C'1	C'2	C'3	C'4	PROPERDIN (UNITS)	Mg	Ca	AGGLUTININ FOR ZYMOSAN (TITER)
Serum	+	+	+	+	+ (5)	+	+	+ (1:16)
Serum + Na_3 EDTA	+	+	+	+	+ (5)	0	0	+
Serum + Na_2 Mg EDTA	+	+	+	+	+ (5)	+	0	+
Serum + Na_2 Ca EDTA	+	+	+	+	+ (5)	+	+	+
Heated serum (56° C./30 min.)	0	0	±	+	0 (< 1)	+	+	+
End piece (R1)	0	+	+	+	0 (< 1)	+	+	+
Mid-piece (R2)	+	0	+	+	+ (7)	+	+	0
R3 (made with *S. dysenteriae*)	+	+	0	+	0 (< 1)	+	+	+ (1:16)
R4	+	+	+	0	+	+	+	+
Fraction II	0	0	0	0	0 (< 1)	+	+	+
RP (made with *S. dysenteriae*)	+	+	+	+	0 (< 1)	+	+	+ (1:16)

This was further tested by the known requirement for Mg^{++} in the interaction of properdin with zymosan (Table V).[1, 12] Agglutinin could be demonstrated in the absence of divalent cations, and in all mixtures it could be removed completely from the serum by zymosan. However, in those mixtures lacking Mg^{++} (containing Na_3EDTA or Na_2Ba EDTA) no measurable properdin was adsorbed to the zymosan. This was demonstrated in these experiments by showing that the C'3 titer after incubation of the zymosan button in RP remained unchanged. Thus the adsorption of antibody to zymosan was not apparently related to the adsorption of properdin to zymosan, and the two reactions could be differentiated by ion requirements.

An attempt was made also to remove the agglutinin from serum by adsorption with zymosan at 0° C., a temperature at which interaction with properdin is minimal.[1] The properdin titer of the serum should, under these circumstances, remain unchanged. This form of adsorption, however, removes from normal serum a factor distinct from both agglutinin and properdin[13] which is necessary for the inactivation of C'3 by the properdin-zymosan complex and affects the

TABLE V. EFFECT OF IONS ON AGGLUTININ FOR ZYMOSAN AND PROPERDIN ACTIVITY AFTER INCUBATION OF SERUM WITH ZYMOSAN*

SERUM WITH 0.015M	IONS LACKING	AGGLUTININ FOR ZYMOSAN		ACTIVITY OF PZ RESIDUAL C'3 IN RP (UNITS)
		BEFORE INCUBATION WITH ZYMOSAN	AFTER INCUBATION WITH ZYMOSAN	
Na_3 EDTA	Ca, Mg	+ (1:16)	0	80
Na_2Ba EDTA	Ca, Mg	+ (1:16)	0	80
Na_2Mg EDTA	Ca	+ (1:16)	0	10
Na_2Ca EDTA	—	+ (1:16)	0	20 (control 80)

*Either Na_3EDTA, Na_2Ba EDTA, Na_2Mg EDTA, or Na_2Ca EDTA was added to serum in 0.015M concentration and the zymosan agglutinin titer was determined. The serum mixtures were then incubated with 10 mg. of zymosan per milliliter for 60 minutes at 17° C. The zymosan with any adsorbed protein was removed by centrifugation and the agglutinin titer of the supernatant was redetermined. The zymosan pellet was washed twice with cold saline, added to an RP (in a concentration of 7 mg. per milliliter) and incubated (37° C. for 60 minutes) to determine whether properdin had been adsorbed to the zymosan by the inactivation of C'3.

assay of properdin by the zymosan procedure. Accordingly, the zymosan agglutinin could be readily adsorbed from serum at 0° C. with zymosan, but the zymosan had little or no adsorbed active properdin after such treatment since it would not inactivate C'3 in RP. Other data[14] indicate that active properdin cannot be eluted from zymosan treated in this manner. However, the properdin titers on the adsorbed serum were variable and uninterpretable, presumably because of changes in this other factor. Thus, under the circumstances of these experiments, the relationship between properdin and agglutinin to zymosan suggested by Nelson[11] could not be shown. Properdin could be removed from serum without affecting agglutinin content, and agglutinin could be removed by adsorption to zymosan without adsorbing properdin to the zymosan.

These experiments and experiments on partially purified properdin[12] clearly distinguish properdin from agglutinins to zymosan and show that the agglutination phenomenon, like most antigen-antibody interactions, is independent of complement and divalent cations.

DISCUSSION

The description of the components and properties of the properdin system has been in vitro.[1, 15-17] However, the potential biologic importance of the properdin system lies in its possible functions in in vivo systems, particularly if a role in natural defense mechanisms can be demonstrated. In vitro the demonstration of properdin involves the interaction between serum and zymosan which results in the formation of the properdin-zymosan complex and the inactivation of the third component of complement.[1] Properdin also combines with a variety of other high molecular weight polysaccharides[2] some of which also cause inactivation of the third component. It is possible that the role of properdin in its various interactions in vitro may be the result of the interaction of properdin with polysaccharide constituents of the particular cellular elements involved, whether they are red cells, bacteria, or virus particles.

In vivo, properdin might be expected to interact with the same high molecular weight polysaccharides with which it combines in vitro. Pillemer and Ross[18] have already observed that zymosan administered parenterally causes marked reduction in properdin levels in rats and mice. The observations reported here indicate that properdin combines with zymosan and other polysaccharides in rabbits and that there is a close correlation between the in vitro and in vivo properties of polysaccharides for combination with properdin. Only those reagents with in vitro combining properties caused a reduction in the serum properdin level after injection into the rabbit.

After intravenous administration, and depending on dose and agent, the level remained low for variable periods up to 2 weeks. A "rebound" to high levels of properdin, observed in rats and mice, occurred only inconstantly.[18] Parenteral administration of zymosan also caused inactivation of C'3, an observation which differs from the experience in rats in which no alteration of C'3 occurred. However, others have noted change in total complement after administration of zymosan to rats, a change presumably in C'3.[27]

In vivo, zymosan and other polysaccharides cause fever and leukopenia, typical of the bacterial pyrogenic responses. Properdin interacts with a number of endotoxic materials derived from gram-negative organisms.[2] It may be argued, then, that the properdin combining properties of zymosan are actually due to endotoxic properties of zymosan or to bacterial contamination of the zymosan preparations during their production; indeed, Shilo and Wolman[19] have demonstrated contamination of native levans with endotoxin. There is, however, a lack of correlation between endotoxic properties and properties related to interaction with properdin as follows: (1) lethal doses of bacterial endotoxin did not cause a fall in properdin level; (2) agents active and inactive against properdin had similar pyrogenic properties; (3) doses of zymosan 100 times as great as those causing marked fever and a decrease in properdin level were not lethal; (4) not all agents that caused a decrease in properdin level caused the typical endotoxic response with fever and leukopenia; and (5) zymosan failed to serve as the preparatory dose for either the local or generalized Shwartzman reaction, a property it should possess if its activities were due to endotoxic properties. Thus it is difficult to attribute the results in these experiments to contamination with endotoxin.

One interesting aspect to the present study is the apparent relation between the ability of agents to combine with properdin and to precipitate the local Shwartzman reaction. Bennett has previously demonstrated that native dextrans are capable of eliciting the local Shwartzman reaction.[20] The studies here were limited, in that only a small number of agents were tested. The findings here are in contrast to those of Arndt and co-workers[21] who were unable to demonstrate precipitation of the local Shwartzman reaction with zymosan in either rabbits or mice using the same zymosan (5B171) used in the present studies. The reason for the discrepancy is unknown. As was pointed out, the observed effects were probably not due to endotoxic properties, for zymosan does not serve as a preparatory reagent for the Shwartzman reaction; multiple doses of zymosan or zymosan in combination with bacterial endotoxin do not cause kidney lesions characteristic of the general Shwartzman reaction; and the production of both the local and the generalized Shwartzman reaction by bacterial endotoxin are not associated with a change in serum properdin level.

The spontaneous occurrence in the serum of normal human subjects of antibodies to zymosan, a yeast derivative, is not surprising and is similar to the observations of Nelson and Lebrun[22] on the natural occurrence of antibodies to starch granules in normal human serum. The ubiquity of yeasts makes likely frequent contact with yeasts, and antibodies to them could develop.

The development in animals of antibodies to zymosan after single or multiple injections was expected. The heterogeneity of zymosan preparations and the complex constitution of zymosan with both carbohydrate and protein components makes it likely it could be antigenic.[9]

Nelson has challenged the existence of the properdin system as a unique entity and has suggested that properdin is an antibody to zymosan. The bases for his argument are: (1) that the inactivation of C'3 by zymosan is not specific but also occurs in classic complement fixation by antigen-antibody aggregates;

and, (2) that normal human serum and preparations of purified properdin contain agglutinating antibodies to zymosan. Discussion of the first point does not relate to the data contained in this present report.

The data in this report relate directly to the second point, the relationship between properdin and agglutinins to zymosan. These current data indicate clearly the dissociation between agglutinin and properdin in serum on the basis of their complete separation in different fractions of serum, their specific inactivation by different procedures, and their independent removal by cross absorption studies. Pernis[23] has made similar observations. This distinction in serum is similar to the differentiation between properdin and the agglutinin for zymosan in partially purified human properdin.[12] Thus, although agglutinins for zymosan do exist in serum they are clearly distinct from properdin. Although antibodies to zymosan were demonstrated by Nelson, he reported no assays for properdin or other experimental evidence which would serve to establish the identity or distinction of properdin and antibody to zymosan.

The possible relation of properdin to resistance has been suggested on the basis of correlative studies demonstrating changes in resistance and properdin levels after the administration of zymosan, bacterial endotoxin, or other polysaccharides. Demonstration of cause and effect relationship is difficult. The premise of such experiments is that the effect of an agent such as zymosan or bacterial endotoxin is due solely to its interaction with properdin. That this may not be correct is suggested by the variety of biologic effects noted here. Zymosan is neither a single substance nor of uniform composition. Administration of zymosan has both humoral and cellular effects as demonstrated here and by others.[24-26] Thus any in vivo studies on the properdin system and its relation to natural immunity based on administration of such polysaccharides must be interpreted with caution.

SUMMARY

1. The injection into rabbits of zymosan and other high molecular weight polysaccharides caused a fall in properdin level and C'3 level in serum.

2. The ability to produce these effects in vivo correlated with the ability of these agents to combine with properdin in vitro.

3. The injection of zymosan and dextran produced fever and leukopenia similar to that caused by endotoxin, but the properdin level of the recipient animal did not affect pyrogenic or leukopenic responses.

4. Zymosan and those dextrans which interact with properdin were effective in provoking the local Shwartzman reaction, but did not serve as the preparatory agent for the local Shwartzman reaction.

5. The properties for combination with properdin are probably not directly related to endotoxic properties inherent in or contaminating the polysaccharide preparations.

6. Injection of zymosan into rabbits caused the appearance of an antibody to zymosan. Some human sera spontaneously contained agglutinins for zymosan.

7. The antibody to zymosan could be clearly distinguished from properdin itself.

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A SYMPATHETIC BLOCKING AGENT, BRETYLIUM TOSYLATE (DARENTHIN), IN THE TREATMENT OF HYPERTENSION

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Bretylium tosylate can, by blocking the sympathetic nervous system, achieve a similar degree of the control of blood pressure as is possible with ganglion blocking agents. It does not produce the distressing side effects due to blockade of the parasympathetic system seen with the ganglion blocking drugs. It does, however, suffer from some of the other disadvantages of ganglion blocking agents in that individual titration of dosage is required and many patients develop tolerance.

THE introduction of a new agent, bretylium tosylate,[1] which selectively inhibits sympathetic nervous activity without affecting parasympathetic function, presents the possibility of achieving similar control of blood pressure as was obtained with ganglion blocking agents but without the unpleasant side effects. This report attempts to define the place that this sympathetic blocking agent might have in the treatment of hypertension and specifically compares its effects on the patient with those obtained by various ganglion blocking drugs in current use.

MATERIAL AND METHODS

Blood pressure records, obtained either in the hospital or by twice daily readings taken by the patient in the home, serve as the basis for estimation of the degree of blood pressure control achieved by the new drug. Thirty patients with hypertension were studied. Bretylium† was administered in 3 equal doses with meals, starting with 100 to 200 mg. three times daily and increasing the dosage every 1 to 2 days by 100 mg. thrice daily until the desired effect was achieved or side effects appeared.

In fifteen patients bretylium was used as the initial antihypertensive agent either alone (seven patients) or in combination with chlorothiazide. Another fifteen subjects undergoing treatment for severe hypertension with ganglion blocking drugs were converted to bretylium in order to compare the blood pressure control achieved by these two agents. All these patients had been taking chlorothiazide and this was continued. To achieve the change-over from one drug to the other each patient was asked to halve the dose of the ganglion blocking agent and to start with the new drug in a dosage level of 200 mg. thrice daily. After 3 days he was asked to increase the bretylium by 100 mg. three times a day for a further 3 day

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TABLE I

PATIENT	DIAGNOSIS	AGE (YR.)	BLOOD PRESSURE BEFORE TREATMENT		FINAL DOSE (MG./DAY)	BLOOD PRESSURE WHILE ON BRETYLIUM TOSYLATE		DURATION (DAYS)	REMARKS
			RECUMBENT (MM. HG)	STANDING (MM. HG)		RECUMBENT (MM. HG)	STANDING (MM. HG)		
1	Essential hypertension	50	200/120	190/120	1,500	200/110	155/100	5	Nil
2	Essential hypertension	44	145/100	145/100	1,600	145/105	135/100	6	Nil
3	Malignant essential hypertension	40	280/150		(Hydralazine 200) 900*	240/150	180/110	10	Tolerance
4	Primary aldosteronism	45	210/130	200/125	600	190/105	160/ 90	13	Dizziness on standing
5	Essential hypertension; cerebrovascular disease	47	220/150	190/120	1,500	200/125	160/110	14	Transient elevation of blood pressure initially
6	Malignant essential hypertension	45	180/120	176/132	600*	164/104	145/ 90	15	Nil
7	Essential hypertension; cerebrovascular disease; ischemic heart disease	46	170/120	155/110	600*	160/110	140/100	16	Tolerance
8	Renal hypertension (pyelonephritis)	38	200/130		600*	200/130	160/120	16	Nausea, elevation of blood pressure with initial dose intravenously
9	Renal hypertension (chronic nephritis)	23	220/160	190/150	1,100	186/133	167/132	30	Headaches, tolerance
10	Essential hypertension	39	208/129	206/114	(Reserpine 0.5) 1,200*	211/114	217/120	30	Postural syncope
11	Renal hypertension (chronic nephritis)	43	172/124	176/130	600	149/114	158/128	30	Headaches, dizziness on standing, indigestion, tolerance
12	Essential hypertension	38	192/118	186/123	500*	155/100	140/100	30	Nasal stuffiness
13	Essential hypertension	44	202/121	212/125	1,500*	178/ 95	174/ 94	50	Nil
14	Essential hypertension	54	180/110	160/110	900	191/108	143/ 92	61	Nasal stuffiness, conjunctival injection, tolerance
15	Essential hypertension	46	214/109	214/131	1,200*	156/ 90	133/ 89	67	Conjunctival injection, indigestion, tolerance
	Averages	43	200/126	185/122	Bretylium tosylate average 993	182/113	158/105	26	

In Patients 1 to 12 treatment was initiated in the hospital and the posttreatment level was also recorded in the hospital for Patients 1 to 8 and with home blood pressure readings for Patients 9 to 12. In Patients 13 to 15 all blood pressure records before and after treatment are average of readings taken twice daily at home.

One case of severe hypertension and primary aldosteronism[4] was controlled with bretylium for a short period while necessary diagnostic studies were being made.

*Patients who started chlorothiazide simultaneously (1 Gm. per day).

period. In this manner the dosage was increased until the level of the standing blood pressure was the same as had been previously achieved with the ganglion blocking agent. At this point the latter drug was to be omitted completely and the dose of bretylium readjusted until the control of pressure was the same as that which had been achieved before. Since we considered that a potentially harmful upsurge of pressure might occur during the change from one drug to the next, patients were instructed to return to their previous medication if their systolic pressure on standing exceeded 200 mm. Hg on 3 successive observations.

RESULTS

A. *Treatment With Bretylium Tosylate, ab Initio.*—In fifteen patients bretylium tosylate was used as the initial antihypertensive agent. Since the objective of this part of the study was to observe the effects of the new drug, most of the patients were studied in the hospital. In eight patients, chlorothiazide was started simultaneously with the bretylium. The average control blood pressure in the fifteen subjects was 200/126 mm. Hg in the recumbent position and 185/122 mm. Hg in the standing position. Bretylium tosylate in a dosage range of 600 to 1,600 mg. per day was effective mainly in lowering the blood pressure with the patient in the standing position, the average level after treatment being 158/105 mm. Hg whereas the average pressure with the patient in the recumbent position was 182/113 mm. Hg (Table I, Fig. 1). Further review of the details of the blood pressure records in the hospital made it evident that the effect on the orthostatic blood pressure was greater in the morning than in the evening.

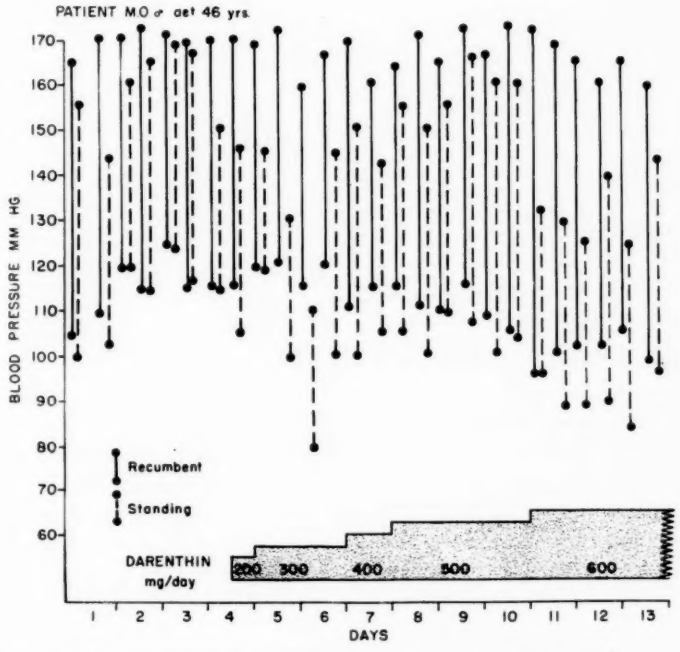

Fig. 1.—The effect of bretylium on the standing and recumbent blood pressure showing the early development of tolerance.

*Patients who started chlorothiazide simultaneously (1 Gm. per day)

TABLE II. CONVERSION OF PATIENTS FROM

PATIENT	DIAGNOSIS	AGE (YR.)	BLOOD PRESSURE BEFORE TREATMENT		TREATMENT
			RECUMBENT (MM. HG)	STANDING (MM. HG)	
1	Malignant essential hypertension; ischemic heart disease; renal failure	57	234/148	232/150	Mecamylamine
2	Essential hypertension	55	251/146	270/170	Mecamylamine
3	Renal hypertension; chronic nephritis	28	202/130	190/140	Mecamylamine
4	Essential hypertension; cerebrovascular disease; ischemic heart disease	50	266/146	210/130	Mecamylamine
5	Essential hypertension	33	188/127	212/154	Mecamylamine
6	Malignant essential hypertension	49	200/125	180/125	Mecamylamine
7	Essential hypertension (splanchnicectomy); ischemic heart disease	40	210/130	210/130	Mecamylamine
8	Essential hypertension	37	260/170	284/148	Mecamylamine
9	Essential hypertension	39	201/115	174/124	Mecamylamine
10	Renal hypertension	28	170/118	178/123	Mecamylamine
11	Essential hypertension (splanchnicectomy)	29	196/128	178/135	Mecamylamine (Reserpine
12	Essential hypertension	26	165/111	172/114	(Reserpine Mecamylamine
13	Essential hypertension; ischemic heart disease	46	200/100	204/108	(Hydralazine Mecamylamine
14	Essential hypertension	49	218/133	230/130	(Pempidine
15	Renal hypertension	52	194/124	142/120	(Trimethidinium
Averages		41	210/130	204/133	Mecamylamine

Blood pressure readings on treatment are the average of recordings made twice daily by the patient at home. The average of the readings was taken for 5 days before the latest clinic visit.

All these patients were receiving chlorothiazide (1 Gm. per day) throughout the period of study.

The side effects produced by the drug were not severe, the most common being dizziness on standing, nasal stuffiness, and conjunctival injection. Three patients complained of vague abdominal discomfort (Table I). A rise in blood pressure was seen at the initiation of treatment in 2 patients. One of these followed an intravenous administration of the drug, but in the other it appeared 6 hours after starting oral medication and was quite alarming, reaching a maximum of 290/150 mm. Hg. Six patients in this group showed clear evidence of the development of tolerance over the first 2 weeks on bretylium. The details in one case are presented in Fig. 1. These patients responded initially to quite a small dose of bretylium, 300 to 600 mg. per day, but slowly required additional amounts of drug to regain control of pressure. However, after the first 3 weeks no further increases in dosage have been required. At this time, however, two patients had apparently become refractory to the drug.

GANGLION BLOCKERS TO BRETYLIUM TOSYLATE

FINAL DOSE (MG./DAY)	BLOOD PRESSURE WHILE ON OTHER DRUGS		FINAL DOSE (MG./DAY)	BLOOD PRESSURE WHILE ON BRETYLIUM TOSYLATE		DURATION (DAYS)
	RECUMBENT (MM. HG)	STANDING (MM. HG)		RECUMBENT (MM. HG)	STANDING (MM. HG)	
10	188/127	151/110	1,600	205/134	154/120	124
15	186/110	152/120	1,200	213/112	164/100	56
50	120/ 90	130/100	2,400	147/104	153/110	45
30	213/109	146/ 86	2,400	194/ 99	114/ 67	80
40	180/114	193/130	2,800	182/113	188/124	106
10	195/112	160/100	800	180/109	148/ 90	125
20		154/ 95	900	148/ 88	147/ 88	125
15	232/128	148/ 92	2,600	233/121	156/107	24
30	164/117	97/ 68	900	160/110	102/ 70	22
15	138/111	128/111	1,200	146/113	128/100	55
20 0.5)	198/151	148/121	1,000 (Reserpine 0.5)	207/156	159/120	122
0.25) 20	171/104	133/ 99	(Reserpine 0.25) 3,000	180/108	169/104	10
600) 55	190/ 97	172/ 96	(Hydralazine 600) 2,400	186/ 90	180/ 95	16
10)	190/108	178/108	1,200	198/105	168/ 87	92
120)	195/116	175/119	1,200	194/115	164/108	66
25	183/114	151/104	1,707	185/112	153/ 99	71

In two patients, not included in this table, the conversion to bretylium tosylate was not successful. One patient noticed injection of the conjunctiva, headache, and extrasystoles; the other also had a severe headache. He noticed his pressure to be very high and following instructions returned to his previous medication.

B. *Comparative Study With Ganglion Blocking Agents and Bretylium Tosylate.*—Fifteen patients with severe hypertension who had been treated for many months with chlorothiazide and a ganglion blocking agent were chosen for conversion to bretylium tosylate (Table II). For the most part these patients were receiving mecamylamine in a dosage range of 10 to 40 mg. per day. One was receiving trimethidinium, 120 mg. per day, and another pempidine, 10 mg. per day. Three patients were also receiving a third drug: one took hydralazine, 600 mg. per day, and two reserpine, 0.5 mg. per day, and these drugs were continued during the period of conversion.

The initial blood pressure of these patients averaged 210/130 mm. Hg recumbent and 204/133 mm. Hg standing. This cannot be strictly regarded as a control level since it is separated by a considerable period of time, 2½ to 64 months, from the level recorded under ganglion blocking therapy. It is used to

indicate the initial severity of the disease in each patient. The average level of pressure after ganglionic blockade was 183/114 mm. Hg recumbent and 151/104 mm. Hg standing. With no other changes in their management apart from the substitution of bretylium tosylate for the ganglion blocking agent the average level of blood pressure was practically unchanged in the recumbent position (185/112 mm. Hg) and on standing (153/99 mm. Hg). The average dose of bretylium tosylate was 1,707 mg. per day with a range from 800 to 3,000 mg. per day. This is to be compared with an average dose of 25 mg. of mecamylamine daily. Sensitivity to the ganglion blocking drug was not reflected in a similar sensitivity to bretylium. For example, of two patients, each taking 10 mg. of mecamylamine per day, one required 800 mg. and the other 1,600 mg. of bretylium per day (Nos. 1 and 6, Table II). Furthermore the patient (No. 11) whose blood pressure was uncontrolled on 3,000 mg. per day of bretylium had responded adequately to a moderate dose of mecamylamine, 20 mg. per day.

The most striking feature of the conversion to bretylium was the almost complete relief from the side effects caused by parasympathetic blockade. Dry mouth, constipation, and blurred vision ceased and two patients noted that impotence was lessened. Three patients complained of nasal stuffiness and two of injection of the conjunctiva. Having been on ganglion blockers for many months the patients were pleased with the freedom from side effects caused by the new agent; such minimal discomfort as they noted was of little consequence. However, two patients not included in Table II suffered such severe side effects from bretylium that attempts at conversion from ganglion blockers were abandoned. One complained of conjunctival injection, headache, and frequent extrasystoles. The other also experienced a severe headache and found his systolic blood pressure to be 240 mm. Hg. He therefore resumed the former dose of mecamylamine.

Unlike our experience with the first group only two of these patients who had previously had ganglion blocking drugs showed clear evidence of tolerance to bretylium.

DISCUSSION

Our observations indicate that the effect of bretylium tosylate on the blood pressure is practically identical with that obtained with ganglion blocking agents, and this is achieved with very few side effects. Although the new drug appears to possess definite therapeutic advantages it is still far from an ideal antihypertensive drug. In the first place, differences in individual sensitivity to its effects and its relatively short period of action necessitate careful titration of dosage if effective control of blood pressure is to be maintained. Furthermore, the hypotensive effect is exhibited mainly with the patient in the standing position. We have noticed that the postural effect is more marked in the morning, perhaps because of greater absorption of the drug overnight and for this reason patients should be particularly careful in the bathroom in the morning. We have not noticed excessive tachycardia on standing and this is probably due to the fact that this agent, unlike former sympatholytic drugs, blocks the sympathetic nerves to the heart.

An important disadvantage exists in the absence of any means to estimate the degree of blockade achieved at any moment. With ganglion blocking agents the pupillary light reflexes are a useful guide to the effective dose.

The use of the word tolerance in connection with this drug and with ganglion blockers is not an ideal term since it probably does not refer to a true failure in response of the tissue but may be caused by the blood vessels readjusting their resistance when deprived of their normal sympathetic tone, much as they do after sympathectomy. This interpretation seemed likely since only slight evidence of tolerance appeared in patients who had previously been under the effect of ganglion blockade, whereas in those in whom bretylium was used as the first blocking agent tolerance occurred more frequently. Since careful adjustment of dosage is only required in the initial stages of treatment the development of tolerance has not been a serious handicap. Patients who become completely refractory to the drug continue to respond to ganglion blocking agents.

Although bretylium will become a very important antihypertensive drug, the disadvantages which have been noted suggest that its place will be, as ganglion blocking agents are now, in the treatment of patients with severe forms of hypertension and in whom other forms of therapy such as chlorothiazide or reserpine have been unable to bring the blood pressure into a relatively safe range.

SUMMARY

The control of blood pressure by bretylium tosylate has been studied in thirty hypertensive patients. Half of these had never received blocking drugs previously and in the remaining fifteen a direct comparison was made of the effects of bretylium with those of ganglion blocking agents.

The main effect of bretylium was upon the pressure with the patient in the standing position which was effectively reduced by a dose of 600 to 2,800 mg. per day. The side effects, which consisted of nasal stuffiness, conjunctival injection, vague abdominal discomfort, and postural dizziness, were not severe. Tolerance developed within the first 3 weeks of treatment in half of the patients not previously on blocking drugs.

The comparative studies of ganglion blocking drugs with bretylium showed that, whereas the control of blood pressure was practically identical with the 2 drugs, there was complete relief from parasympathetic side effects with the latter agent.

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PROTEINS IN NORMAL AND NEPHROTIC SERA WITH FILTER PAPER ELECTROPHORETIC MOBILITY OF ALBUMIN

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Serum protein components can be studied in greater detail with the aid of new electrophoretic techniques (starch gel electrophoresis and immunoelectrophoresis).

In our studies the homogeneity of human albumin in normal and nephrotic sera as well as nephrotic urines was investigated by these methods. Proteins were found which migrate with the mobility of albumin when subjected to electrophoresis in filter paper but which migrate with lesser mobilities when subjected to electrophoresis in starch gel.

These proteins can be subdivided into two major groups. Their distribution in the sera studied is given and some of their physicochemical characteristics are demonstrated and discussed.

THE electrophoretic fraction referred to generally as albumin has recently been shown not to be homogeneous by several workers. Hughes[1-3] describes the presence of mercaptalbumin. Hoch-Ligeti and Hoch[4] found from 2 to 4 albumin peaks in diluted human sera during prolonged electrophoresis in phosphate buffer (pH 8.0, ionic strength 0.1). When human albumin is subjected to moving boundary electrophoresis in buffers of low ionic strength and low pH, two boundaries were observed by Luetscher,[5] and four by Saifer and Corey.[6] Miller and collaborators[7] used acetate buffer of low pH (3.9) but of high ionic strength (0.2, 90 per cent NaCl) and demonstrated four components in human serum albumin. Heterogeneity of human albumin was also shown by chromatography on anion-exchange cellulose[8] and anion-exchange resin.[9] Larson and Feinberg[10] have shown heterogeneity of human serum albumin by immunologic methods. Knedel[11] and recently Gitlin[12] and Earle and associates[13] have demonstrated and inherited anomaly displaying two albumin peaks by free boundary and filter paper electrophoresis.

During our studies of serum protein distribution in the nephrotic syndrome of children and other hypoproteinemias, proteins were observed which had the mobility of albumin by filter paper electrophoresis but which, when subjected to electrophoresis in starch gel in a discontinuous system of buffers, migrated with a lesser mobility than that of albumin. The serum of one of the nephrotic children contained such proteins in unusually high concentrations and a series of four protein zones which migrate with progressively less mobility in the

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gel than in normal albumin were readily seen.[14, 15] Recently Fraser and coauthors[16] described an albumin-like protein the presence of which is possibly determined genetically. This report describes the number, distribution, and some of the properties of several proteins which can be separated from the albumin with the aid of electrophoresis in starch gel.

METHODS AND MATERIALS

Sixty-four sera of normal individuals (all age groups) and 32 sera of children afflicted with active juvenile nephrosis were investigated in this study. Urines, collected at admission from sixteen of these patients and concentrated approximately 20 times under reduced pressure in collodion bags (Schleicher and Schuell), were also studied. Urines of five nephritic children were concentrated in the same way. A serum rich in albumin-like protein was used in comparative and immunologic work.

All sera and urines were subjected to two dimensional zone electrophoresis[17] in the discontinuous system of buffers.[18] The electrophoresis in the first dimension (filter paper) was conducted under rigidly standardized conditions. For this purpose the moist chamber type apparatus, with the filter paper strips suspended horizontally, was placed in a hood, and after applying 0.1 ml. of the materials on Whatman 3MM paper (1½ inch strip), the electrophoresis was conducted at a constant temperature of 30° C. for 16 hours at a voltage gradient of 3 v. per centimeter. The buffer used was composed of 11.04 Gm. sodium barbital and 3.75 Gm. sodium acetate (anhydrous) dissolved in 1 L. of distilled water. Under these conditions a complete separation of albumin from α_1-globulin was always achieved. Many of the sera were also subjected to electrophoresis in other buffers, previously described.[17] Highly specific antiserum, prepared in rabbits using a crystalline albumin as antigen, was donated by Dr. William Oliver (Department of Pediatrics, University of Michigan Medical School, Ann Arbor).

RESULTS

Our experiments indicate that several proteins exist which have a filter paper electrophoretic mobility close to that of albumin but which have different mobilities in starch gel. For the sake of description, they will be divided into two groups. Proteins characteristic of the first group have been shown to be immunologically related to albumin.[15] This group of proteins will be referred to as the albumin-like proteins. One of these has been observed in about 50 per cent of normal individuals. This component migrates on the filter paper with the same mobility as albumin; its amount is variable in the normal sera and is usually quite small. In starch gels its mobility is nearly equivalent to that of transferrin-C.[16] Fig. 1 shows results of the two dimensional zone electrophoretic and immunoelectrophoretic experiments performed on the serum in which this protein is present in readily demonstrable quantity (serum of Dr. Harris). Its relationship to albumin is clearly demonstrated. The immunologic relationship between albumin and the albumin-like protein was further studied by the single diffusion method of Oudin,[19, 20] the double diffusion method of Ouchterlony,[21] and by the filter paper electrophoretic method of Poulik.[22] Both the single and the double diffusion methods gave one precipitin line. When the albumin portion of the filter paper electrophoresis strip of the serum that contained both the normal and the albumin-like protein was subjected to antigen-antibody reaction in agar gel (filter paper immunoelectrophoresis), again only one precipitin line developed. This result is shown in

Fig. 2. The two dimensional zone electrophoretic and immunoelectrophoretic methods proved particularly suitable for the present investigations.[15-17] Using these techniques the described albumin-like protein was readily demonstrable in twenty-four of the sixty-four normal individuals screened. In the sera of eleven of the remaining forty individuals, the amount of this protein was so small that its presence was classified as "probable." Whether this protein was completely absent in the remaining twenty-nine individuals or whether the amount present was too small to be detected with the currently used methods remains to be seen.

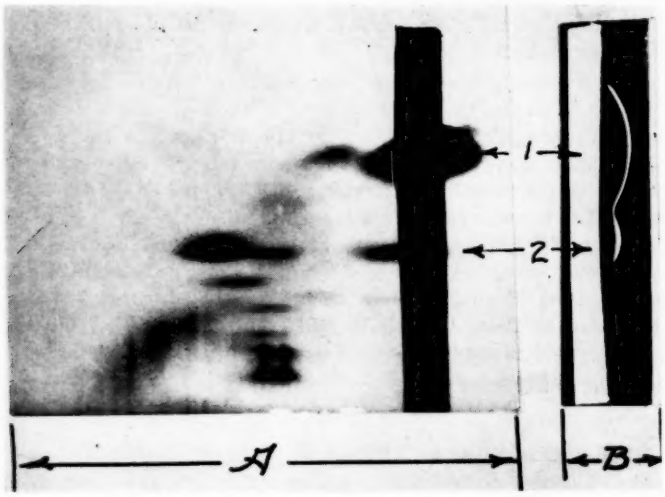

Fig. 1.—Demonstration of albumin-like protein in a normal serum. *A*, Two dimensional zone electrophoretic pattern of normal serum. Starch block was dissected from the albumin portion of the gel. *B*, Contact prints of the precipitin lines appearing after 30 hours over starch block. *1*, Albumin; *2*, albumin-like component.

In contrast to these findings in normal sera, we find that this protein is almost always absent in sera of nephrotic children (31 sera of 32 lacked this protein). On the other hand, this component was found in large quantities in all of the 16 urine samples from the nephrotic patients which were studied. Fig. 3 shows an example of the two dimensional zone electrophoresis and immunoelectrophoresis of one of the urine samples. The presence of the albumin-like protein and its immunologic relationship to albumin is clearly seen. This protein was not found in any one of the five nephritic urine samples screened.

The second group of proteins which we have observed in the majority of cases comprises several protein components which have a filter paper mobility similar to but not identical with that of albumin. These proteins differ from the albumin-like proteins in several respects. (a) On filter paper their mean electrophoretic mobility is less than albumin. They are found mainly under the "slow portion" of the albumin and may in certain cases extend into the α_1-globulin-albumin region. (b) In starch gel the zones tend to be curved in a characteristic way in contrast to the albumin-like proteins which do not curve. (c) They

appear to be unrelated to albumin immunologically. (d) Their presence or absence in a given serum seems to be unrelated to the presence or absence of albumin-like protein(s). This group includes probably some of the postalbumins of Smithies[23] but other new proteins undoubtedly belong to this group as well. They seem to belong to a definite group electrophoretically (filter paper). Since their number is variable (0 to 6 found in our series) they may not be functionally related. They will be referred to as quasialbumins. The distribution of these

Fig. 2.—Filter paper immunoelectrophoretic result obtained with a normal serum that contained normal albumin and albumin-like protein. Contact print of the precipitin line appearing after 30 hours over filter paper.

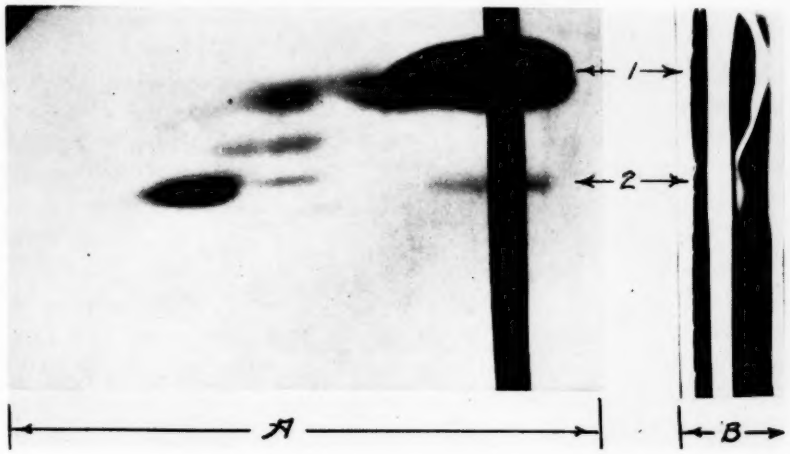

Fig. 3.—Demonstration of albumin-like protein in nephrotic urine. *A*, Two dimensional zone electrophoresis of a nephrotic urine (concentration, approximately 20 times). Starch block was dissected from the albumin portion of the gel. *B*, Contact print of the precipitin lines appearing after 32 hours over starch block 1. *1*, Albumin; *2*, albumin-like component.

proteins is shown in Fig. 4. Their presence was found in 61 of the 64 normal sera screened and in 26 of the 32 sera of nephrotic children. They were not found in any one of the nephrotic urine samples but were found in the nephritic urine samples. Their presence was always more easily detected in sera from nephrotic patients than in those from normal individuals (regardless of age). It is of interest that they could easily be observed in sera from two individuals subjected to plasmaphoresis (2 patients studied).

Proteins of either or both the two groups observed may be present or absent in serum from a given individual. Fig. 5 shows diagrammatically the three possible combinations of the occurrence of these proteins. However, individuals who lacked both the albumin-like and the quasialbumin proteins were also found. The following distribution of such groups was found in our series of normal

individuals: albumin-like only, three individuals; quasialbumins, twenty-eight individuals; both groups, thirty-two individuals; neither group, one individual. The distribution among the nephrotic patients was as follows: albumin-like, one patient; quasialbumins, twenty-six patients; both groups, no patient (since the albumin-like protein is lost in the urine); neither group, five patients.

Fig. 4.—Distribution of quasialbumins in a normal serum (albumin and α_1-globulin regions of a two dimensional zone electrophoresis pattern are shown).

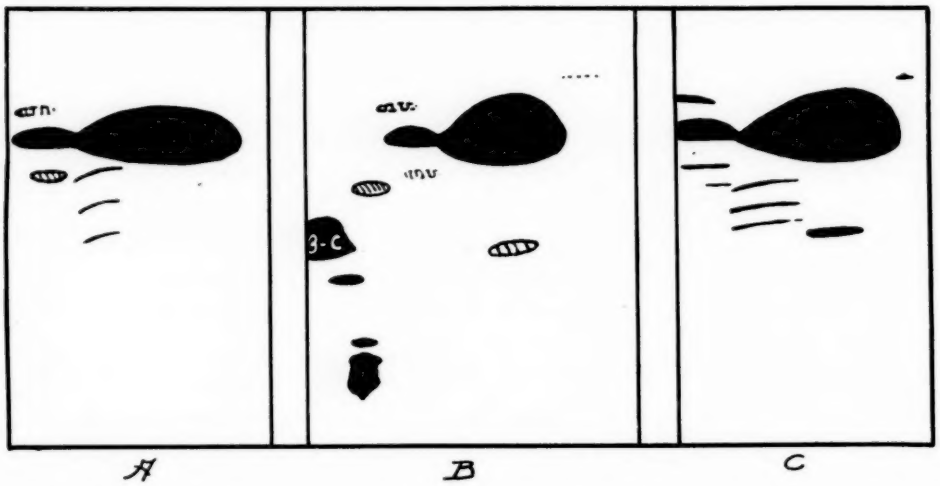

Fig. 5.—Diagrams (traced from photographs) of the distribution of albumin-like and quasialbumin proteins in normal individuals. *A*, Quasialbumins, *B*, albumin-like protein; *C*, quasialbumins and albumin-like protein.

The existence of several albumin-like proteins may be the result of polymerization of albumin molecules, possibly through a disulfide (—S—S—) bond. A normal serum, rich in the albumin-like protein was subjected to electrophoresis in a gel prepared in the tris-citric acid buffer to which was added 0.01M 2-mercaptoethanol. The albumin-like zone was then no longer demonstrable. Thus the presence of a disulfide link in the albumin-like protein is possible.

DISCUSSION

The presence of proteins which may have the same electrophoretic mobility on filter paper but different mobility(ies) in the starch gel and still be immunologically related was accidentally discovered in a case of a nephrotic child (a 9-year-old girl). The first routinely drawn specimen (obtained 1½ years ago) showed the typical pathologic distribution of the serum proteins. However, investigation of the serum obtained 3 months later, at the height of the disease, showed several albumin-like proteins present in unusually high amounts. Most of the same proteins were also found in the urine and ascitic fluid obtained on the same date. The immunologic relationship of these proteins to albumin could be established by immunoelectrophoresis.[15] In retrospect the first specimen was again examined and this time slight traces of similar proteins could be detected since the supposed artefacts found on first examination were now seen in a different perspective. Unfortunately for various reasons this case could not be followed at regular intervals. The patient was under treatment and refractory to therapy and did not respond well. A third specimen of serum was recently obtained when her clinical state was somewhat improved but still abnormal. This time the proteins found were of the quasialbumin group only. The same group of proteins was also found in the sera of the parents and in one sibling.

The biologic functions of the albumin-like proteins and the quasialbumins are unknown to us. It is possible that some of the latter proteins may be inhibitors of chymotrypsin or peptidases, which have been reported to migrate with a mobility between α_1-globulin and albumin.[24, 25] These proteins are present not only in varying numbers in a given individual but also in varying concentrations in the same person.

A more detailed and specific classification of both these protein groups is in progress. Such a classification should make it possible to establish whether these proteins are under genetic control. Several families with identical and nonidentical twins were studied with respect to inheritance of these proteins. These preliminary studies showed a surprising (perhaps fortuitous) result, namely that the albumin-like protein was observed in all members of the families with the identical twins (both parents and twins). In the families with nonidentical twins, such findings were less constant. A larger series of such families is under study.

SUMMARY

Two groups of proteins were found in normal and nephrotic sera which have the filter paper electrophoresis mobility of albumin but which have different mobilities in starch gel. Protein of one of the groups is also related to albumin immunologically. One of these proteins was invariably found in the urine

samples from nephrotic individuals. The proteins of the second group belong to a distinct group on filter paper which can be separated into several proteins by electrophoresis in starch gel. These proteins were found in the sera of the majority of normal and nephrotic individuals.

The distributions of the groups (and their variants) are given.

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CARBOHYDRATE INTERMEDIATES IN HEPATIC CIRRHOSIS: ROLE OF LIPOIC ACID

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Pyruvate and lactate metabolism has been investigated in twenty-one patients with hepatic cirrhosis. Nineteen patients had chronic alcoholism. Despite adequate doses of thiamine pyruvate metabolism was abnormal in twenty patients; lactate metabolism was disturbed in seventeen. Twenty patients were treated with intramuscular lipoic acid (6,8 dithio-octanoic acid), 30 mg. daily for 3 days; the tests were then repeated. Studied as a whole the average figures for pyruvate and lactate metabolism were not influenced by treatment with lipoic acid. Studied individually there was improvement in pyruvate and lactate metabolism in three patients, but this was not statistically significant. There was a corresponding deterioration in four patients but this was not associated with change in the clinical condition. In two patients there was a remarkable fall in the blood concentrations of pyruvate and lactate after oral or intravenous glucose; this was not corrected by lipoic acid but may have been due in part to preceding malnutrition. It is concluded that lipoic acid does not favorably influence disturbed pyruvate or lactate metabolism in patients with hepatic cirrhosis and is therefore unlikely to be of value in the therapy of hepatic coma.

IT has been known for many years that the concentrations of lactate and pyruvate are raised in the blood of patients with liver disease[1-4] and an abnormal response to a glucose load has also been reported in patients with hepatic cirrhosis; the raised blood pyruvate after such a load varied directly with the severity of the injury to the liver.[5]

Among its important metabolic roles in normal subjects pyruvate occurs as an intermediate in the metabolism of glucose to carbon dioxide and water via the Krebs cycle. In the presence of an oxygen deficit in the tissues, it is reduced to lactic acid, the final stable product of anaerobic glycolysis.[6] The oxidation of one molecule of glucose to two of lactate yields 2 high reactivity phosphate bonds, whereas complete oxidation to carbon dioxide and water yields a further 36 such bonds[7] emphasizing the importance of complete pyruvate utilization in the energy supply of the body.

For pyruvate to enter the Krebs cycle it must first be oxidatively decarboxylated and this reaction requires diphosphopyridine nucleotide (DPN), reduced coenzyme A (Co A SH), magnesium ions, thiamine pyrophosphate (TPP), and lipoic acid (6,8 dithio-octanoic acid). The following scheme for the reaction has been proposed by Reed[8]:

1. Pyruvic acid + TPP $\rightleftharpoons$ acetyl TPP + CO_2.

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TABLE I

CASE NO.	SEX	AGE (YR.)	ALCOHOLISM	VASCULAR SPIDER	ASCITES	PROTHROMBIN CONCENTRATION (%)	SERUM BILIRUBIN (MG. %)	SERUM FLOCCULATION TESTS	REMARKS
1	F	48	0	–	+	30	24.0	3+	Not alcoholic
2	M	58	+	–	+	73	2.1	1+	Last exposure to alcohol 6 weeks previously
3	F	45	+	+	+	100	1.6	2+	Last exposure to alcohol 4 weeks previously
4	F	27	0	+	+	14	5.6	3+	Impending coma—prednisone 10 mg./day
5	M	38	+	–	+	15	13.2	3+	Alcohol to time of admission—died later. Protein-free diet
6	M	65	+	+	–	40	21.0	3+	Alcohol 3 months previously. Prednisone 20 mg./day
7	M	45	+	+	+	13	22.9	3+	Alcohol 1 month previously. Prednisone 10 mg./day
8	F	41	+	+	+	40	10.4	1+	Alcohol 1 week previously
9	F	57	+	+	–	80	0.8	2+	Alcohol 3 months previously. Hematemesis and impending coma between first and second test; protein-free diet
10	M	48	+	+	–	32	2.1	2+	Alcohol 1 week previously—impending coma
11	M	38	+	+	+	26	4.0	3+	Alcohol 3 weeks previously
12	M	51	+	–	+	58	13.5	3+	Alcohol 1 month previously
13	F	56	+	–	+	100	2.0	2+	Alcohol 3 months previously
14	M	45	+	–	–	78	1.0	3+	Alcohol 6 weeks previously
15	F	41	+	–	–	58	1.1	3+	Alcohol 6 weeks previously
16	F	35	+	+	+	8	15.0	2+	Alcohol 2 months previously
17	M	42	+	–	–	20	25.0	2+	Alcohol 3 months previously
18	M	40	+	–	+	69	2.5	–	Alcohol 1 month previously
19	F	30	+	–	–	43	1.6	2+	Alcohol 3 weeks previously
20	M	57	+	–	+	58	2.0	3+	Alcohol 2 weeks previously
21	M		+	+	+	30	2.3	3+	Alcoholism, acute fatty liver, protein-free diet. Intravenous glucose

2. Acetyl TPP + lipoic acids ⇌ acetyl lipoic acid + TPP.
3. Acetyl lipoic acid + Co A SH ⇌ acetyl Co A + reduced lipoic acid.
4. Reduced lipoic acid + DPN ⇌ lipoic acid + DPNH.

Subsequently the acetyl Co A formed in reaction 3 condenses with oxaloacetate to form citrate and the cycle is then completed according to the steps suggested by Krebs.[9] A similar series of reactions to those described above is probably required for the oxidation of ketoglutarate to succinyl Co A and succinate at a later stage in the cycle.

The increased concentration of pyruvate in the blood of patients with cirrhosis could result either from a failure of incorporation of the keto acid into the Krebs cycle by the peripheral tissues, from a reduced utilization of pyruvate by the damaged liver, or a combination of both factors. Deficiency of thiamine is not responsible and supply of an excess of this vitamin will not correct the abnormality. Alternatively the accumulation of pyruvate could result from a deficiency of lipoic acid, although at present there is no syndrome known to result from a shortage of this compound. Moreover it is by no means certain that the body is dependent on exogenous supplies; more probably its synthetic abilities are sufficient to meet normal daily needs, but this may not be the case in the presence of liver damage. Certain recent reports have attributed improvement in the clinical condition of patients in hepatic coma to treatment with lipoic acid,[10-12] the mechanism being, presumably, the improved utilization of carbohydrate by the brain and other tissues. Others have not been able to confirm these observations.[13] However, there are no large series of patients studied with well-controlled biochemical observations and the effect of lipoic acid has been observed only on fasting or random concentrations of blood pyruvate.

We decided, therefore, to reinvestigate the effect of lipoic acid on the ability of patients with hepatic cirrhosis to metabolize pyruvate and lactate after a glucose load and in the presence of adequate doses of thiamine. As there is no known "dose" of lipoic acid* we gave 30 mg. daily by intramuscular injection and although this amount may be inadequate it is rather more than has been used by most other workers in this field.

METHODS

Clinical Material.—Twenty-one patients with chronic liver disease were investigated. All were admitted to the medical wards of the Jose Joaquin Aguirre Hospital, Santiago, Chile. The majority were seen in the Gastroenterological Unit. Of these twenty-one patients nineteen had chronic alcoholism, i.e., they drank wine in liter quantities daily. Four had been drinking to within a few days of admission; because of deteriorating health the remainder had not taken alcohol for from 1 to 3 months previously. Two patients gave no history of alcoholism, but had had preceding episodes of hepatitis. Details of these twenty-one patients can be found in Table I.

Pyruvate and Lactate Metabolism Tests.—Except where otherwise stated all patients were on a normal ward diet which supplied between 60 and 70 Gm. protein daily. Certain

*Lipoic acid, Lederle Laboratories, Inc., 10 mg. per milliliter.

of the patients with cirrhosis were on protein-free diets because of impending hepatic coma; for comparison two control subjects were also studied after 5 days of protein withdrawal. All patients were given 100 mg. thiamine intramuscularly for 3 days before the tests were performed. The pyruvate metabolism test was carried out according to the method of Joiner and co-workers,[14] glucose being given in two doses of 50 Gm. at zero time and 30 minutes. Blood was drawn by venipuncture from the antecubital fossa at 0, 60, and 90 minutes.

Pyruvate was estimated by the method of Friedman and Haugen[15] and lactate according to the method of Barker and Summerson.[16]

After the base-line response of blood pyruvate and lactate to the glucose load had been determined the patients were all given 30 mg. lipoic acid intramuscularly daily for 3 days and the test was repeated one hour after the last dose.

Twenty patients were studied for the effect of lipoic acid on the pyruvate and lactate tolerance.

One patient was investigated after intravenous glucose had been received in a dose of 0.33 Gm. per kilogram.

Control Observations.—These were made on patients with normal liver function as follows: two patients with arthritis were studied before and after protein withdrawal and two obese patients on low calorie diets were investigated during a period of rapid weight loss.

RESULTS

Pyruvate and Lactate Metabolism.—In the initial tests nineteen of the twenty patients gave concentrations of pyruvate in the blood above the upper limits of normal set by Joiner and associates[14] while the remaining patient was on the upper normal limits. The mean figures for the twenty patients together with the range of variation and the standard deviation are given in Table II, and these are compared with the normal results quoted by Joiner and collaborators. The average figures for lactate metabolism are above the normal values given by Henneman and Bunker[17] though four fall on the upper normal limits. These results for the twenty patients are given in Table III and are compared with the normal figures of Henneman and Bunker.

TABLE II. EFFECT OF LIPOIC ACID ON PYRUVATE METABOLISM IN PATIENTS WITH HEPATIC CIRRHOSIS (PYRUVATE AS MG./100 ML.)

	BEFORE LIPOIC ACID			AFTER LIPOIC ACID		
1. 20 Patients						
Time (min.)	0	60	90	0	60	90
Mean	1.61	2.09	2.31	1.66	1.95	2.25
Maximum	3.14	3.10	3.65	3.48	3.40	3.64
Minimum	0.79	1.23	1.33	0.82	1.21	1.47
S.D.	0.61	0.71	0.58	0.71	0.60	0.57
*2. 50 Normal Subjects**						
Mean	0.76	0.92	0.94			
S.E.	0.18	0.17	0.20			

*Joiner and co-workers[14] considered that results should be regarded as abnormal if they exceeded the normal mean + 2 S.E., i.e., 1.12 mg., 1.26 mg., and 1.34 mg./100 ml. at 0, 60, and 90 minutes, respectively.

Thus all but one patient, despite adequate diet and thiamine, gave abnormal results.

After 3 days' treatment with lipoic acid (30 mg. per day) the tests were repeated. Although individual patients showed marked changes the mean figures were remarkably similar. These are to be found in Table II for pyruvate and Table III for lactate and are also illustrated graphically in Fig. 1.

TABLE III. EFFECT OF LIPOIC ACID ON LACTATE METABOLISM IN PATIENTS WITH HEPATIC CIRRHOSIS (BLOOD LACTATE MG./100 ML.)

	BEFORE LIPOIC ACID			AFTER LIPOIC ACID		
1. 20 Patients						
Time (min.)	0	60	90	0	60	90
Mean	11.6	15.6	17.7	13.2	15.8	17.4
Maximum	32.1	25.8	30.9	38.8	35.8	42.5
Minimum	4.5	8.8	8.9	2.3	4.9	10.4
S.D.	6.1	3.3	5.1	4.4	6.9	7.5
*2. Normal Subjects**						
Mean	7.0	12.0	9.6			
S.D.	0.5	0.4	0.5			

*Henneman and Bunker.[17]

Despite the failure of the average figures to show any variation between those obtained before and those after treatment with lipoic acid certain individual patients deserve further attention. Four patients showed a marked deterioration in both pyruvate and lactate metabolism after lipoic acid (Fig. 2). One of these patients suffered a severe hematemesis between the first and second study but there was no such episode in any of the other three to account for the change in metabolism. Furthermore, withdrawal of the lipoic acid resulted in a return of the fasting levels to pretreatment concentrations.

In contrast to these four patients three showed improvement and their results are shown in Fig. 3. It will be seen from this that although the fasting and 60 minute figures for both pyruvate and lactate are improved the 90 minute results show less change, particularly in respect to lactate. Furthermore, the metabolism tests are still very abnormal (Fig. 3).

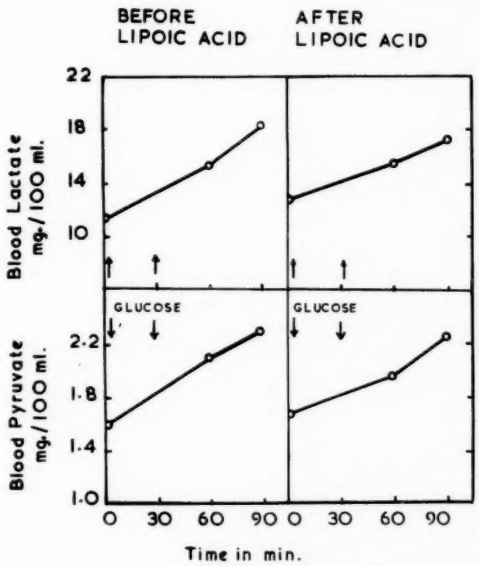

Fig. 1.—Changes in blood pyruvate and lactate after oral ingestion of glucose. Mean figures from twenty patients with hepatic cirrhosis. On the left before, on the right after treatment with lipoic acid, 30 mg. daily for 3 days.

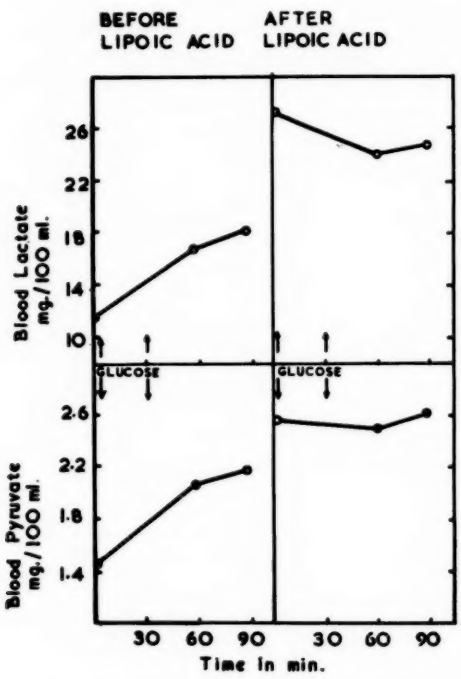

Fig. 2.—Apparent worsening in pyruvate and lactate metabolism after treatment with lipoic acid, 30 mg. daily for 3 days; mean figures for four patients with hepatic cirrhosis.

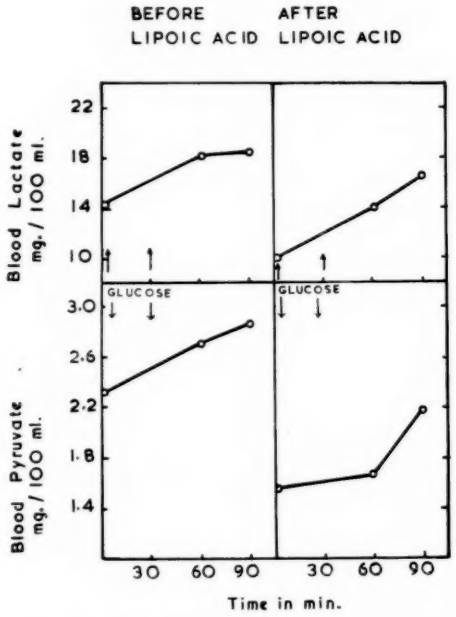

Fig. 3.—Apparent improvement in pyruvate and lactate metabolism after treatment with lipoic acid, 30 mg. daily for 3 days; mean figures for three patients with hepatic cirrhosis.

Two patients deserve particular mention because they showed very high fasting levels for both pyruvate and lactate which fell precipitately after the glucose load. Both patients were severely ill and were on protein-free diets; neither had received lipoic acid at the time of these tests. One patient (Case 5) subsequently was given lipoic acid which did not alter the abnormal shape of

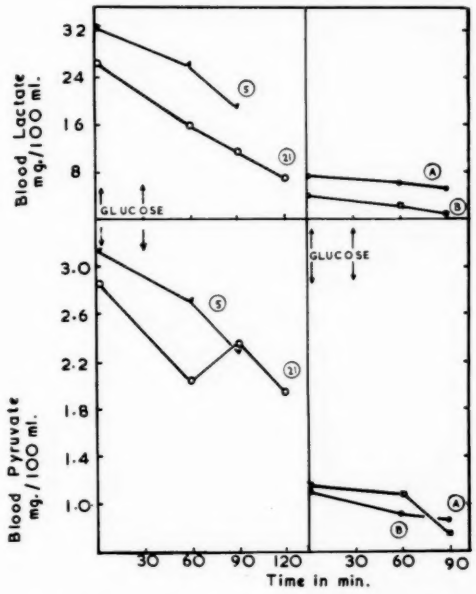

Fig. 4.—Pyruvate and lactate metabolism during calorie restriction. *A*, Two patients with hepatic cirrhosis and impending hepatic coma; *B*, two obese control subjects.

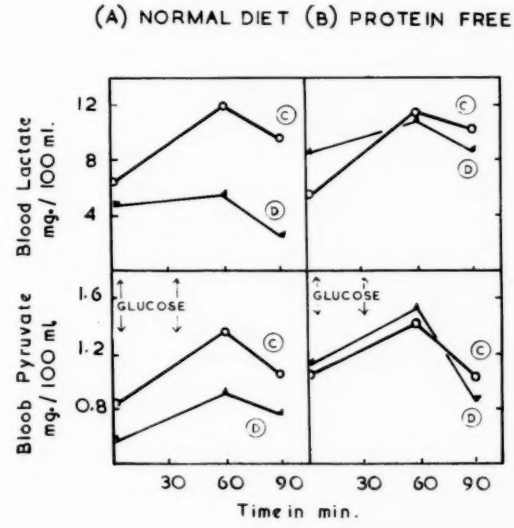

Fig. 5.—Effect of protein withdrawal on pyruvate and lactate metabolism on two obese control subjects.

the tolerance curve, but did cause a general lowering of the concentrations of both pyruvate and lactate at all three time intervals. The second patient (Case 21) was given a glucose load intravenously in order to rule out the possibility that an intestinal absorption defect was causing the abnormal metabolism. The results are shown in Fig. 4 and, for convenience, are compared with similar tests carried out on two obese patients on severely restricted diets; both these patients were losing weight rapidly at the time. This shows that inadequate calorie intake and weight loss in an otherwise normal individual is also associated with a "falling" curve for both pyruvate and lactate although the actual figures were within the normal range. Protein withdrawal alone, associated with an otherwise normal calorie intake, did not result in this type of falling curve (Fig. 5).

DISCUSSION

Comparison of the average figures for the pyruvate and lactate metabolism tests before and after treatment with lipoic acid show clearly that there was no change in the pattern of carbohydrate utilization that could be ascribed to the action of lipoic acid. Similarly there was no change in the clinical condition of any of the patients.

An attempt to break down the figures into subgroups was hardly more enlightening. Although, in three patients the "after treatment" results were lower than the initial levels, they were still well outside the normal range (Fig. 3). Against these three patients who showed improved metabolism must be set the four who showed gross deterioration and only in one of these could we find an obvious explanation. As the mean fasting levels of both pyruvate and lactate returned rapidly to pretreatment levels after lipoic acid was discontinued it is difficult not to attibute the change to the action of the compound. Altschule and collaborators[18] also found that prolonged treatment with very large doses of lipoic acid caused biochemical and clinical deterioration in certain cases of chronic schizophrenia. They suggested that this was due to a relative deficiency of thiamine in the face of excess lipoic acid, an explanation which is hardly valid in our study.

An attempt to break down the figures on clinical grounds (i.e., alcoholic and nonalcoholic or prednisone treated and untreated) was equally unsatisfactory and gave no useful information. Finally, mention must be made of the two patients who had the remarkable "falling curves" for both pyruvate and lactate (Fig. 5). Clinically these men had both been drinking heavily up to the time of admission to the hospital and both were severely ill; they would seem to fit into the category described by Phillips and Davidson[19] as having "acute hepatic insufficiency of the chronic alcoholic." This group has a particularly bad prognosis and indeed one of our two patients died. One possible explanation of these falling curves is that the sudden glucose load supplied a much needed energy source to a failing liver and enabled it to take up excess pyruvate for glycogen synthesis. Certainly both these patients had been on grossly deficient diets with alcohol as the main, if not the only, calorie

source. While we do not really accept this explanation, we have no other to offer. Figs. 4 and 5 illustrate the effect of calorie restriction and protein withdrawal in the normal subject. A period on a low calorie intake prior to the test did seem to cause a change in both pyruvate and lactate metabolism similar to that observed in the two patients, although of a much less severe degree. It is therefore possible that this type of falling curve is simply a reflection of a preceding episode of malnutrition which is greatly exaggerated by coincident liver damage.

SUMMARY AND CONCLUSIONS

Twenty-one patients with chronic liver disease (nineteen alcoholic) have been studied for abnormalities of metabolism of some carbohydrate intermediates. Pyruvate metabolism was abnormal in twenty patients and lactate metabolism was abnormal in seventeen despite preliminary saturation with large doses of thiamine.

Twenty of these patients were then treated with lipoic acid, 30 mg. daily for 3 days, and the test was repeated. The average figures for both pyruvate and lactate metabolism showed no change after treatment. In 4 cases there appeared to be a deterioration in the biochemical findings which was rapidly corrected after lipoic acid was discontinued and therefore suggested a causative relationship. In all cases changes in blood lactate levels during the test closely followed those in pyruvate and there was no evidence of a dissociation in the metabolism of these two compounds.

There was no evidence that lipoic acid favorably influenced the disturbed carbohydrate metabolism of chronic hepatic disease in the doses given and similarly there was no evidence of any change in the clinical condition of the patients.

Two patients showed a remarkable fall in concentration of both pyruvate and lactate after glucose and some evidence was adduced that this might have been in part related to a previous state of malnutrition.

We wish to thank Dr. H. S. Stutz of Lederle Laboratories, Inc., for the lipoic acid and one of us (J. M. W.) is indebted to The British Council who initiated and sponsored his visit to Santiago.

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INCIDENCE OF LEPTOSPIRAL AGGLUTININS IN PATIENTS

A Ten Year Experience

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During the period from 1950 to 1959 serum specimens from 521 patients in general hospitals have been examined for agglutinins for 8 leptospiral serotypes. Titers of 1:200 or higher were observed with thirty-one (6 per cent) of the patients. The serologic evidence obtained indicated that thirteen of the patients were probably infected with Leptospira canicola, twelve with Leptospira pomona, and six with Leptospira grippotyphosa. The agglutinin titers are correlated with the time of onset of symptoms. Positive reactions were obtained as early as 4 days after onset in two patients and a negative reaction as late as 13 days in one patient. Heterologous reactions are discussed. The results obtained illustrate the value of the agglutination test in the diagnosis of leptospirosis in patients.

CASES of leptospirosis in humans are being recognized with increasing frequency in the United States. Practical methods for the laboratory diagnosis of the disease have been described adequately[1-3] and the clinical aspects of leptospirosis recently have been reviewed.[2, 4, 15] This report summarizes the results obtained in the examination of specimens from over 500 patients whose sera were referred to this laboratory during the past decade for the detection of leptospiral agglutinins.

MATERIALS AND METHODS

The patients included in this study were mostly hospitalized patients in whom leptospirosis was either clinically suspected or for whom the possibility of leptospirosis was to be eliminated. Many of the latter had jaundice of unknown etiology. Two hundred and ninety of these patients were seen at the Parkland Memorial Hospital in Dallas, a city-county hospital, 140 at the Veterans Administration Hospital at McKinney, Texas, 31 at the Children's Hospital of Texas, 27 at the Veterans Administration Hospital in Dallas, and the remainder were private patients and patients at other local hospitals.

Sera were examined by a modification of the agglutination-lysis test[1] by adding 0.1 ml. of suspension of live leptospirae to 0.1 ml. of patient's serum dilution. Leptospirae were taken from 1- to 2-week-old cultures in Korthof's medium. Depending upon the density of growth the cultures were diluted 1:2 or 1:3 in sterile medium. After incubation at room temperature for 3 hours a loopful of each mixture was examined microscopically without a coverslip using a 16 mm. objective and darkfield illumination. The highest dilution of serum that showed definite agglutination as compared with a control was taken as the titer. Each serum, in a final dilution of 1:100, was first tested against 8 serotypes. No agglutination in this dilution of serum was considered a negative result. Any leptospiral type which reacted in this preliminary test was then tested against serial doubled dilutions of the serum to

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determine the titer. The serotypes included were *Leptospira icterohaemorrhagiae,** *L. canicola,** *L. pomona,** *L. grippotyphosa,** *Leptospira bataviae,*† *Leptospira hebdomadis,*† and *Leptospira autumnalis* A and AB.†

RESULTS

Table I presents a summary of the results obtained over a period from 1950 through 1959 during which serum specimens from 521 patients were examined for leptospiral agglutinins. All patients included in the tabulation had at least one specimen with a titer of 1:200 or higher for at least one serotype. The classification of the patients according to serotype is presumptive only since it is based on serologic evidence alone except in one patient from whom *L. pomona* was isolated. Altogether, thirty-one patients (6 per cent) showed positive reactions but there was a marked difference in the proportion of positive reactions among patients at Parkland Hospital as compared with patients at the McKinney Veterans Administration Hospital. The former showed 3.8 per cent positive and the latter 11.8 per cent. Patients at McKinney from whom specimens were received represent a more select group with respect to the probability of leptospirosis. In addition, the McKinney Hospital has a higher proportion of patients from rural areas in contrast to the Parkland patients who are predominantly urban residents. This same epidemiologic difference is also reflected in the fact that eight of the patients with *L. canicola,* two with *L. pomona* and one with *L. grippotyphosa* were from Parkland whereas one with *L. canicola,* ten with *L. pomona,* and five with *L. grippotyphosa* were from McKinney where patients would be more likely to come in contact with cattle and other farm animals. No patients were encountered who appeared to have been infected with *L. icterohaemorrhagiae* and one fifth of the patients who showed positive reactions had significant titers for *L. grippotyphosa,* a type not yet isolated from man in the United States.

The agglutinin titers obtained with individual specimens with relation to the time of onset of symptoms are shown in Tables II, III, and V. The marked variability of the results is immediately apparent. Only four patients showed titers of 1:12,800 or higher (Nos. 85, 234, 261, and 406) whereas at least one specimen from twenty-one patients showed a titer of 1:1,600 or higher. The earliest positive reactions were obtained 4 days after the onset of symptoms in patients Nos. 234 and 327. On the other hand, patients Nos. 261, 336, and 339 were negative in 5 days, No. 343 was negative in 7 days, and No. 363 was negative in 13 days. No patient became negative during the period of observation. A significant fall in titer was noted in patient No. 406 in 5 weeks, in No. 139 in 6 weeks, in Nos. 234 and 343 in 4 months, and in patient No. 11 in 6 months. Patient No. 336 maintained a titer of 1 : 3,200 for 2 months.

The serum of patients listed in Table II reacted most strongly with *L. canicola* or with this type alone (Nos. 462 and 500). Titers of these two were among the lowest. Ten of the thirteen patients showed reactions with *L. icterohaemorrhagiae.* Several of these titers are higher than would be predicted from

*The original cultures were supplied by Dr. P. B. Beeson, currently at Yale University School of Medicine.

†Obtained from Dr. E. H. Thomas, National Microbiological Institute, Bethesda, Md.

TABLE I. RESULTS OBTAINED WITH 521 PATIENTS EXAMINED SEROLOGICALLY FOR EVIDENCE OF LEPTOSPIROSIS

YEAR	NO. OF PATIENTS TESTED	NO. OF PATIENTS SEROLOGICALLY POSITIVE			
		L. CANICOLA	L. POMONA	L. GRIPPOTYPHOSA	TOTAL
1950	8	–	2	–	2
1951	33	–	–	1	1
1952	30	2	–	–	2
1953	21	1	–	–	1
1954	75	–	1	1	2
1955	89	3	1	–	4
1956	53	1	–	–	1
1957	56	2	5	1	8
1958	83	1	2	3	6
1959	73	3	1	–	4
Total	521	13	12	6	31

TABLE II. SERUM AGGLUTININ TITERS OF PATIENTS PRESUMABLY INFECTED WITH *L. Canicola*

PATIENT (NO.)	TIME AFTER ONSET	LEPTOSPIRA SEROTYPE*					
		ICTEROHAEMORRHAGIAE	CANICOLA	POMONA	HEBDOMADIS	AUTUMNALIS A	AUTUMNALIS AB
58	6 days	100	400	–†	–	–	–
	4 weeks	100	800	–	–	–	–
67	2 weeks	800	400	–	–	400	–
	3 weeks	800	800	–	–	400	–
	4 weeks	200	3,200	–	–	200	–
	5 weeks	200	3,200	–	–	100	–
85	1 week	–	–	–	–	100	–
	2 weeks	–	800	–	–	100	–
	3 weeks	–	3,200	–	–	100	–
	4 weeks	–	12,800	–	–	–	–
217	19 days	800	1,600	–	–	–	–
	27 days	1,600	3,200	–	–	–	–
234	4 days	–	100	–	–	–	–
	29 days	–	6,400	–	–	–	–
	37 days	–	12,800	–	–	–	–
	4 months	–	3,200	–	–	–	–
244	5 weeks	–	1,600	–	–	–	–
	6 weeks	100	800	–	–	–	–
261	5 days	–	–	–	–	–	–
	11 days	3,200	12,800	–	–	–	–
312	4 weeks	100	200	–	–	–	–
	5 weeks	–	400	–	–	–	–
328	6 days	–	–	200	200	–	–
	15 days	1,600	1,600	800	800	–	–
	4 weeks	1,600	3,200	100	100	–	–
387	?	100	400	–	–	–	–
	?	100	200	–	–	–	–
462	2 years	–	200	–	–	–	–
494	8 days	200	200	–	–	–	100
	11 days	100	400	–	–	–	400
500	?	–	400	–	–	–	–

*None of these sera in a 1:100 dilution agglutinated *L. grippotyphosa* or *L. bataviae.*
†No agglutination in a 1:100 dilution of serum.

the serologic relation between these types as demonstrated by cross agglutination tests with the serum of immunized rabbits.[1] Two of these patients (Nos. 67 and 328) illustrate the so-called paradoxical reaction[3] in which the heterologous titer is higher during the early stages of the disease. The first specimen from patient No. 328 reacted with heterologous types only. This was the only one of these thirteen patients whose serum reacted with *L. pomona* and *L. hebdomadis.*

Patient No. 234 (Table II) is of special interest. The initial titer was inconclusive and other possible diagnoses were considered. More than 3 weeks elapsed before a second specimen that showed a titer of diagnostic significance was obtained. At this time it was recalled that the patient's wife, No. 244, had recently had a similar illness and tests on her serum revealed a titer of 1:1,600 for *L. canicola.*

The twelve patients who were probably infected with *L. pomona* are listed in Table III. The variability of the agglutination reactions is again apparent

TABLE III. SERUM AGGLUTININ TITERS OF PATIENTS PRESUMABLY INFECTED WITH *L. Pomona*

PATIENT (NO.)	TIME AFTER ONSET	LEPTOSPIRA SEROTYPE*					
		ICTEROHAEMORRHAGIAE	CANICOLA	POMONA	HEBDOMADIS	AUTUMNALIS A	AUTUMNALIS AB
1	1 month	-†	-	800	-	-	-
	6 months	-	-	400	-	-	-
11	3 weeks	200	-	6,400	-	-	-
	6 months	-	-	400	-	-	-
139	14 days	-	-	800	-	-	-
	6 weeks	-	-	200	-	-	-
191	11 days	400	1,600	800	-	400	200
	1 month	400	800	3,200	-	800	400
327	4 days	-	-	-	200	-	-
	15 days	800	100	3,200	6,400	3,200	3,200
	26 days	800	100	3,200	800	3,200	3,200
332	29 days	-	-	400	-	-	200
	35 days	-	-	1,600	-	-	800
336	5 days	-	-	-	-	-	-
	10 days	-	-	3,200	100	-	400
	21 days	-	-	3,200	-	-	400
	2 months	-	-	3,200	-	400	400
339	5 days	-	-	-	-	-	-
	11 days	-	-	1,600	-	-	400
	34 days	-	-	1,600	-	800	400
343	3 days	-	-	-	-	-	-
	7 days	-	-	-	-	-	-
	16 days	100	100	1,600	100	3,200	-
	3 months	-	-	800	-	800	200
	4 months	-	-	400	-	400	-
413	7 days	-	-	800	-	400	-
417	2 weeks	-	-	1,600	-	200	-
	4 weeks	-	-	6,400	-	800	800
488	4 weeks	100	-	800	-	1,600	100
	6 weeks	100	-	800	-	1,600	100

*None of these sera in a 1:100 dilution agglutinated *L. grippotyphosa* or *L. bataviae.*
†No agglutination in a 1:100 dilution of serum.

particularly with respect to heterologous reactions. Patient No. 191 was a veterinarian who may well have had contact with several types of leptospirae which would be consistent with the broad reactivity of his serum. There was a marked tendency for the sera in this group of patients to react with members of the *L. autumnalis* group with titers as high as (No. 327) or higher than (Nos. 343 and 488) the titer for *L. pomona.* The case history of patient No. 327 has been reported elsewhere (Edwards, Case II).[4] A leptospira isolated from this patient on the fourth day of illness was identified by us as *L. pomona.*

Because of the tendency of serum of patient No. 327 to react with types other than *L. pomona,* this patient's serum was compared with the serum of a rabbit immunized with the strain isolated from the patient. A rabbit was given daily intravenous injections of 1 ml. of live culture for 5 days and was bled

TABLE IV. AGGLUTINATION OF LEPTOSPIRAE IN SERUM OF PATIENT 327 AND IN SERUM OF RABBIT IMMUNIZED WITH STRAIN ISOLATED FROM PATIENT

	SERUM		
	PATIENT 327	RABBIT 70	
SEROTYPE	15 DAYS AFTER ONSET	7 DAYS AFTER LAST INJECTION	21 DAYS AFTER LAST INJECTION
Patient 327	6,400	12,800	6,400
icteroheamorrhagiae	800	400	50
canicola	100	800	200
pomona	3,200	12,800	3,200
grippotyphosa	< 100	< 50	< 50
hebdomadis	6,400	50	< 50
bataviae	< 100	800	50
autumnalis A	3,200	50	< 50
autumnalis AB	3,200	400	50

TABLE V. SERUM AGGLUTININ TITERS OF PATIENTS PRESUMABLY INFECTED WITH *L. Grippotyphosa*

PATIENT (NO.)	TIME AFTER ONSET	LEPTOSPIRAL SEROTYPE*				
		ICTEROHAEMORRHAGIAE	CANICOLA	GRIPPOTYPHOSA	AUTUMNALIS A	AUTUMNALIS AB
38	24 days	–†	–	1,600	–	–
	32 days	–	–	1,600	–	–
	45 days	–	–	3,200	–	–
	3 months	–	–	1,600	–	–
99	11 days	–	–	–	100	–
	17 days	–	–	400	1,600	–
	28 days	–	–	1,600	3,200	–
	42 days	–	–	3,200	3,200	–
	70 days	–	100	1,600	1,600	200
330	16 days	–	–	1,600	–	–
	5 weeks	–	–	1,600	–	–
363	13 days	–	–	–	–	–
	22 days	–	–	1,600	–	–
	6 weeks	–	–	1,600	–	–
406	2 weeks	100	1,600	25,600	100	–
	5 weeks	100	400	6,400	–	–
443	3 weeks	–	–	1,600	–	–
	4 weeks	–	–	1,600	–	–

*None of these sera in a 1:100 dilution agglutinated *L. pomona, L. bataviae,* or *L. hebdomadis.*

†No agglutination in a 1:100 dilution of serum.

periodically after the last injection. Table IV compares the titers obtained with the patient's serum and those of the rabbit serum. Essentially the same qualitative spectrum was obtained with both sera; neither serum reacted with *L. grippotyphosa*. The titers of the rabbit serum, however, do not reflect a close antigenic relationship between the patient's strain and either *L. hebdomadis* or *L. autumnalis* A or AB which is suggested by the reactions of the patient's serum. There is an indication that the heterologous reactivity of the rabbit serum diminishes with time. Such a degree of broad reactivity would not have been predicted from the published serologic relationships of type strains.[1, 2]

The case histories of three of the patients listed in Table V have been published elsewhere. Patient No. 38 was the first case of apparent *L. grippotyphosa* infection to be reported in the United States.[6] Patients Nos. 99 and 330 are, respectively, cases VI and VIII of Edwards.[4] There can be little doubt of the infecting type in 4 of these cases since *L. grippotyphosa* was the only serotype agglutinated. This is in contrast to the patients with *L. canicola* and *L. pomona* whose sera showed a greater tendency for heterologous reactions. None of the sera from patients with *L. pomona* or *L. canicola* agglutinated *L. grippotyphosa*.

DISCUSSION

The proportion of positive reactions in this series of tests provides some indication of the prevalence of leptospirosis in man in this area. Since not all of the febrile patients admitted to the hospitals concerned were tested, it is reasonable to suppose that some cases of leptospirosis were missed. During most of the period covered by this study, however, our laboratory was the only one in this area performing tests for leptospirosis and would have received specimens from most of the suspected cases. In view of the relatively high incidence of leptospirosis in dogs,[7] in cattle and swine,[8] and in wild animals[9] one might expect that the disease in man would be observed more often than it is. It may be significant that our lowest percentage (2 per cent) of positive patients occurred in 1956 (Table I), the last of a series of unusually dry years, whereas 1957 showed the highest proportion of cases (14 per cent). This was a season of higher than average rainfall in this area.

It is well known that serologic evidence cannot be taken as a reliable indication of the serotype responsible for the infection. *Leptospira pomona* has been isolated from a cow showing strongest agglutination with *Leptospira sejroe*[10] and *L. pomona* has been isolated from a dog whose serum gave a higher titer with *L. autumnalis* than with *L. pomona*.[11] It seems unlikely, however, that none of the patients listed in Table V was infected with *L. grippotyphosa*. The source of infection in these patients is unknown. The only *L. grippotyphosa* isolations in this country have been from raccoons [9, 10, 12] and foxes[12] although agglutinins have been reported in cattle[10, 14] and dogs.[11] These cases in humans suggest that this type may be prevalent in either wild or domestic animals in this area.

The marked reaction of sera from four patients (Nos. 99, 327, 343, and 488) with *L. autumnalis* A presents the possibility of infection with a member of this group. This seems unlikely, however, for several reasons. In the first place

L. pomona was isolated from one of these patients (No. 327). Second, a member of the *autumnalis* group has not been isolated from man or animals in the United States except in the "Fort Bragg Fever" outbreak.[12, 14] In the study of the Fort Bragg cases[14] it is not stated that the patients' sera showed any marked tendency to react with either *L. pomona* or *L. grippotyphosa.*

SUMMARY

Serum specimens from 521 patients in general hospitals during a period of 10 years have been examined for agglutinins for 8 leptospiral serotypes. Titers of 1:200 or higher were observed with thirty-one (6 per cent) of the patients. As judged from the serologic evidence obtained, thirteen patients were probably infected with *Leptospira canicola,* twelve with *Leptospira pomona,* and six with *Leptospira grippotyphosa.* Correlation of agglutinin titers with the time of onset of symptoms revealed positive reactions as early as 4 days in two patients but the serum of one patient did not become positive until after the thirteenth day. The accumulated results illustrate the value of the agglutination test in the detection of leptospiral infection in patients.

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CITRIC ACID EXCRETION BEFORE AND AFTER CALCIUM INFUSION IN NORMAL AND OSTEOPOROTIC SUBJECTS*

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Renal excretion of calcium and citric acid was measured in a group of twelve normal subjects and twelve osteoporotic patients in 24 hour urines collected after the subjects had been on a constant calcium diet for 5 to 7 days. No statistically significant differences in calcium or citric acid excretion were found between these groups of patients.

Standard calcium infusions were carried out in six normal and nine osteoporotic patients which produced significant hypercalciuria in all patients when calcium excretion on the day preceding and the day of calcium infusion was compared. No significant change in citric acid excretion occurred in either group on the day of calcium infusion. Six osteoporotic and six normal subjects were similarly infused and changes in calcium and citric acid excretion were measured in 1 or 2 hour urine specimens preceding, during, and after the calcium infusions. Despite seven- to fourteenfold increments in urine calcium in the last hour of the infusion and the first postinfusion hour, no significant change in citric acid excretion occurred. These data indicate that no direct relationship exists between urine calcium levels and citric acid excretion.

FEW STUDIES are available which clearly delineate the relationship between urinary calcium and citric acid in normal man. Shorr and co-workers[1] reported that the renal excretion of citric acid was governed by urinary calcium levels in a patient with hyperparathyroidism and on other occasions stressed the importance of urinary calcium concentration in the control of citric acid excretion.[2, 3] Milne[4-6] has presented data which appear to confirm this postulated relationship.

Other reports are at variance with this concept. Ostberg found a lower level of citric acid excretion when urinary calcium was increased by a high calcium diet.[7] Dietrick and associates[2, 8] and later Whedon and Shorr,[9] studying normal men immobilized in body casts for prolonged periods and patients with paralytic poliomyelitis, found either no change or a fall in urinary citrate with concomitant hypercalciuria. Chang and Freeman noted a marked decline in urinary citrate when hypercalciuria was induced in dogs by infusion of calcium chloride[10] and Herrin and Lardinois[11] reported no change in urinary citrate during infusion of calcium gluconate.

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The present study is part of a continuing investigation of the effects of calcium infusion in patients with metabolic bone disease. The changes produced in urine calcium and citric acid excretion by intravenous calcium infusions were studied in a group of patients with postmenopausal or hypogonadal osteoporosis. The results were compared with the changes in urine calcium and citrate observed in normal subjects similarly infused.

MATERIALS AND METHODS

The subjects of this study consisted of twelve patients with osteoporosis and twelve hospitalized patients without renal, hepatic, or skeletal disease who served as controls. Ten of the osteoporotic patients were considered to have postmenopausal osteoporosis and two had osteoporosis associated with primary ovarian failure of the dysgenetic type. These two patients were 35 and 36 years of age at the time of this study and had received no therapy for 12 and 15 years, respectively. The subjects were all maintained with full ambulation on the Metabolic Ward or the General Medical Wards of the Georgetown University Hospital throughout the study period.

The patients on the Metabolic Ward were fed liquid formula diets containing 300, 500, or 1,500 mg. of calcium. Diets for the patients on the General Medical Wards were prepared in a special diet kitchen and contained 150, 300, or 500 mg. of calcium, calculated from standard food tables. Urine specimens were collected for 24 hour periods or for 1 or 2 hour periods prior to, during, and after calcium infusions. These collections were begun after the subject had been on the diet for at least 4 days. Calcium infusions were prepared to contain 10 mg. of calcium per kilogram of body weight as calcium gluconate* in 500 ml. of normal saline. No less than 600 mg. of calcium was administered to any subject over a 3 hour period. All subjects were infused in the postabsorptive state and when hourly urine collections were obtained, food was withheld until termination of the last collection period. Changes in 24 hour excretion of calcium and citrate induced by calcium infusion were measured in six of the normal and in nine osteoporotic subjects. In six subjects of each group urine was collected in 1 or 2 hourly periods for 2 hours preceding, during, and for 2 hours after the infusions so that immediate changes in calcium and citric acid excretion produced by the calcium infusion could be evaluated. Urine calcium levels were determined by the method of Clark and Collip[12] whereas citric acid was measured by the technique of Hess and White.[13] Statistical evaluation was carried out in standard fashion.[14]

RESULTS

The 24 hour urinary excretion of calcium and citric acid in the twelve normal and twelve osteoporotic subjects after 5 to 7 days of a constant calcium intake are shown in Table I and Fig. 1. No correlation was found between citric acid and calcium excretion over a wide range of urine calcium levels in either the normal or osteoporotic subjects. The mean 24 hour urinary calcium excretion in the normal subjects was 161.9 ± 14.7 mg. (S.E.) in 24 hours and 150.0 ± 23 mg. (S.E.) in 24 hours in the osteoporotic group. Citric acid excretion on the control days averaged 462.4 ± 53.9 mg. per 24 hours in the normal subjects and 387.9 ± 40 mg. per 24 hours in the osteoporotic patients. The variations between calcium and citric acid excretion in both groups were not statistically significant, the p value for calcium was greater than 0.5 and that for citrate was greater than 0.1. Calcium excretion of the normal and osteoporotic patients was from 50 to 200 mg. greater in those on 500 mg. diets than was found in

*The calcium gluconate used in this study was supplied by Dr. J. K. Weston of Parke, Davis & Company, Inc.

TABLE I. TWENTY-FOUR HOUR CALCIUM AND CITRATE

NORMAL								
					AVERAGE (MG./24 HR.)		RANGE (MG./24 HR.)	
NO.	SEX	AGE (YR.)	DIET CALCIUM (MG./24 HR.)	NO. DET.	CALCIUM	CITRATE	CALCIUM	CITRATE
1	F	36	150	1	88.0	324.0	—	—
2	F	46	150	3	107.6	517.0	101-112	447-594
3	F	36	1,500	2	114	754.0	108-120	576-932
4	F	17	300	1	121.5	330	—	—
5	F	29	300	1	135.5	678.4	—	—
6	M	41	500	1	158.5	320	—	—
7	M	30	300	1	161	791	—	—
8	M	44	1,500	2	180	292	173-187	270-314
9	M	28	300	1	188.9	376	—	—
10	M	39	500	2	210.5	495	201-220	410-580
11	M	45	500	1	225	290	—	—
12	M	27	500	1	252.2	382	—	—
				Mean	161.9	462.4		
				S.D.	±51.1	±185.7		
				S.E.	±14.7	±53.9		

those on 150 mg. diets. No greater calcium excretion occurred in patients given 1,500 mg. calcium diets than was seen in those on 300 or 500 mg. calcium. Dietary calcium had no apparent effect upon urinary citrate.

Standard calcium infusions were performed in six normal subjects and in nine osteoporotic patients. The average 24 hour urinary calcium excretion in the normal group prior to infusion was 155.5 ± 24.3 mg. per 24 hours. In the osteoporotic patients the mean preinfusion 24 hour calcium excretion was 118.3 ± 20.1 mg. Control day citric acid excretion averaged 511.4 ± 76.6 mg. per 24 hours in the normal group and the osteoporotic patients excreted an average of 387.1 ± 30.6 mg. of citric acid per control 24 hour period.

Calcium infusion produced a significant elevation of urinary calcium in all patients of both groups. The average of the individual increments in urine calcium level (infusion day—control day values) on the infusion day was +287.1 mg. per 24 hours in the normal subjects and +288.5 mg. per 24 hours in the osteoporotic group. The mean of the changes in citric acid excretion of the normal group was –16.3 mg. per 24 hours and this value for the osteoporotic subjects was –24.3 mg. per 24 hours on the day of infusion. There was no statistically significant difference between control day levels of urinary citrate or calcium in these two smaller groups of patients. For the control day values of both calcium and citrate p was greater than 0.1. Comparison of the control day and the infusion day citrate levels in both groups revealed no significant change, despite highly significant elevations in urinary excretion of calcium on the infusion day. Comparing control to infusion days for citrate p was greater than 0.5. Comparing the same days' values in both groups, for calcium p was less than 0.005. The range of citric acid excretion on the infusion day was wide in the normal group. In one subject urinary citrate increased 302 mg. on the infusion day; in two there were decrements of 183.3 mg. and 229.8 mg. while relatively slight changes of +2 mg.–9 mg. and +22 mg. on the day of calcium infusion occurred in the others. A similar range of change in citrate excretion was seen in the osteoporotic subjects on the infusion day. In two subjects increments of 248 and 468 mg. of citrate occurred while significant depressions of

EXCRETION IN NORMAL AND OSTEOPOROTIC SUBJECTS

					OSTEOPOROTIC			
					AVERAGE (MG./24 HR.)		RANGE (MG./24 HR.)	
NO.	SEX	AGE (YR.)	DIET CALCIUM (MG./24 HR.)	NO. DET.	CALCIUM	CITRATE	CALCIUM	CITRATE
1	F	56	150	1	43.7	218.9	—	—
2	F	55	150	2	81.0	444	80-82	338-550
3	F	71	150	2	91.5	513.1	62-121	433-594
4	M	56	300	2	100	206.5	79-121	174-239
5	F	35	300	2	105	346	100-110	324-368
6	M	52	300	1	109.4	416	—	—
7	F	67	300	2	135.5	461	132-139	451-470
8	F	36	300	1	138	341	—	—
9	F	58	500	1	220	450	—	—
10	F	49	500	1	246	390	—	—
11	M	52	500	4	260.5	484	245-273	369-527
12	F	52	500	1	270	385	—	—
				Mean	150	387.9		
				S.D.	±79.1	±117.2		
				S.E.	±23	±40		

222.5, 318.7, 111.5, and 170.8 mg. per 24 hours were found in four. Lesser decrements of 51.9 and 40 mg. were noted in two other osteoporotic subjects. These data are presented in Table II and Figs. 2 and 3, *B*.

In an attempt to exclude the possibility that an acute elevation in citrate excretion followed by compensatory reduction below control levels had been overlooked in the 24 hour measurements, 1 or 2 hourly urine collections were obtained before, during, and after each infusion in six normal and six osteoporotic subjects and were analyzed for calcium and citrate.

TABLE II. CHANGE IN 24 HOUR URINE CALCIUM AND CITRIC ACID LEVELS IN NORMAL AND OSTEOPOROTIC SUBJECTS WITH CALCIUM INFUSION

				CONTROL DAY			INFUSION DAY		CHANGE	
NO.	SEX	AGE (YR.)	DIET	MG. CALCIUM/ 24 HR.	MG. CITRATE/ 24 HR.	MG. CALCIUM INFUSED	MG. CALCIUM/ 24 HR.	MG. CITRATE/ 24 HR.	MG. CALCIUM/ 24 HR.	MG. CITRATE/ 24 HR.
Normal Subjects										
1	F	36	150	88	324.0	600	270.0	326	+182	+2
2	F	46	150	107.6	517.0	600	379.6	333.7	+272	−183.3
3	F	29	150	135.5	678.4	600	291.0	448.6	+155.5	−229.8
4	M	30	300	161	791.0	800	453.0	782.0	+292	−9
5	M	28	300	188.9	376.0	700	733.3	398.0	+544.4	+22
6	M	27	500	252.2	382.0	700	529.0	684.0	+276.8	+302
	Mean			155.5	511.4		442.6	495.2	+287.1	−16.3
	S.D.			±59.6	±187.8		±172.6	±191.9		
	S.E.			±24.3	±76.6		±70.4	±78.4		
Osteoporotic Subjects										
1	F	56	150	43.7	218.9	600	177.4	167.0	+133.7	−51.9
2	F	55	150	81.0	443.7	600	384.9	691.7	+303.9	+248
3	F	71	150	91.2	513.5	600	252.8	291.0	+161.6	−222.5
4	M	56	300	100.5	206.5	700	291.0	187.5	+190.5	−19.0
5	F	35	300	104.8	399.9	600	346.6	229.1	+241.8	−170.8
6	M	56	500	109.4	416.0	700	498.0	97.3	+388.6	−318.7
7	F	67	300	135.9	460.5	700	429.3	349.0	+293.4	−111.5
8	F	36	500	138.0	341.0	700	688.0	809.0	+550	+468.0
9	M	56	500	260.5	484.0	700	593.1	444.0	+332.6	−40.0
	Mean			118.3	387.1		406.7	362.8	+288.5	−24.3
	S.D.			±60.4	±90.3		±164.4	±258.8		
	S.E.			±20.1	±30.6		±54.8	±86.4		

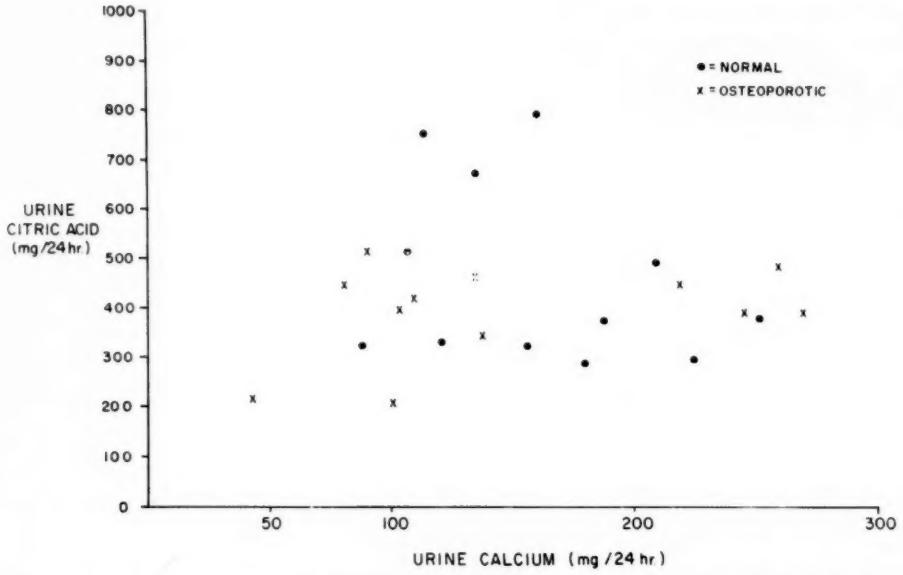

Fig. 1.—Twenty-four hour urine calcium and citric acid excretion in normal and osteoporotic subjects.

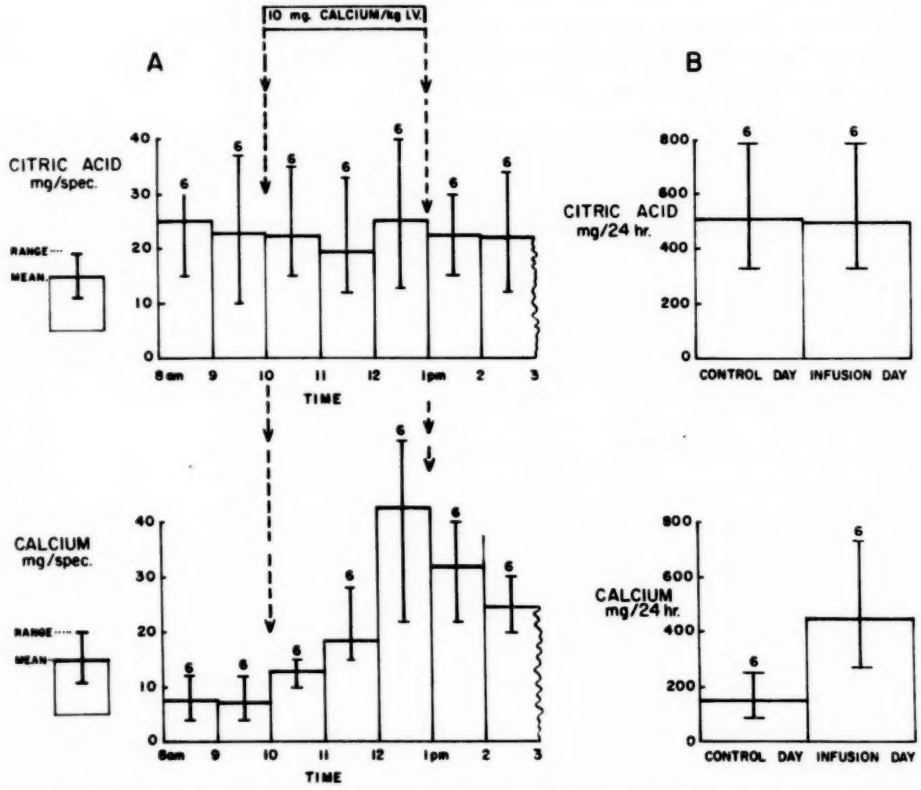

Fig. 2.—Changes in hourly and daily calcium citrate excretion induced by calcium infusion in normal subjects.

TABLE III. ACUTE CHANGES IN URINE CALCIUM AND CITRATE WITH CALCIUM INFUSION

				CONTROL PERIOD				INFUSION PERIOD (10 MG. CALCIUM/KG. I.V.)				POSTINFUSION PERIOD			
TIME OF COLLECTION				8-9 URINE		9-10		10-11		11-12		12-1		1-2	
NO.	SEX	AGE	MG. CALCIUM DIET	CALCIUM MG.	CITRATE SPEC.	CALCIUM	CITRATE	CALCIUM	CITRATE	CALCIUM	CITRATE	CALCIUM	CITRATE	CALCIUM	CITRATE
Normal Subjects															
1	F	36	150	8	30	12	25	15	20	17	12	22	13	31	15
2	F	46	150	5	27	4	30	15	25	20	21	45	35	35	30
3	F	27	150	6	25	8	29	12	15	28	12	40	16	36	18
4	M	52	300	9	15	5	10	10	16	16	12	55	18	40	20
5	M	54	300	12	30	9	37	10	35	15	33	25	40	22	25
6	F	46	500	4	25	5	27	15	25	15	29	40	30	28	28
Mean				7.3	25.3	7.2	23.0	12.8	22.6	18.5	19.8	42.8	25.3	32	22.7
S.D.				±2.9	5.5	3.1	10.6	2.6	7.4	5.01	9.4	13.5	11.2	6.4	5.9
S.E.				±1.2	2.3	1.3	1.8	1.02	3.04	2.04	3.8	5.5	4.5	2.6	2.4
Osteoporotic Subjects															
1	M	56	300	3	8	4	12	12	7	27	5	37	10	30	14
2	F	54	500	4	22	4	22	16	14	16	14	40	22	42	18
3	M	52	500	2	18	2	18	18	12	18	12	55	21	53	23
4	F	71	150	3	18	3	18	20	25	20	25	45	21	40	19
5	F	67	150	5	35	5	35	19	32	19	32	35	30	33	35
6	F	63	300	6	27	6	27	30	18	30	18	59	21	66	19
Mean				3.7	21.3	4.0	22.0	19.2	18	21.7	17.7	45.1	20.8	44.0	21.3
S.D.				±1.5	9.2	1.0	7.8	70.1	9.2	5.5	4.3	10.8	6.6	12.2	6.5
S.E.				±0.6	3.2	0.17	3.2	2.9	3.6	2.2	1.8	4.4	2.8	4.9	2.7

Changes in calcium and citrate excretion are shown for these two groups in Table III and the mean and the range of changes in calcium and citrate excretion are plotted in Figs. 2 and 3, *A*. Despite the production of significant hypercalciuria, greatest in the third hour of the calcium infusion and in the first postinfusion hour, only a transient slight elevation in citrate occurred in four of the normal subjects whereas a minimal increase in urine citrate was noted in three osteoporotic subjects. The mean excretion of citrate fell in the first 2 hours of the infusions in both groups. Statistically, the changes which occurred during infusion and in the first postinfusion hour were highly significant for calcium ($p < 0.001$) and insignificant for citrate ($p > 0.4$) in both the normal and osteoporotic groups. In each group the control specimen with the lowest values for calcium and citrate was used for statistical comparison. In Table IV the statistical values for the groups studied are shown.

DISCUSSION

Among the myriad factors which allegedly influence urinary citric acid concentration, calcium excretion has been afforded recurrent comment but widely divergent opinions continue to be recorded concerning the interrelationships of urinary calcium and citric acid.

The frequently cited work of Milne[4] is the only other study of the effects of intravenous calcium salts on urinary citric acid excretion in man of which we are aware. As part of his doctoral thesis, Milne studied the effects of rapid intravenous infusions of calcium gluconate on citrate excretion using himself

TABLE IV. STATISTICAL DATA

	NONINFUSED CONTROLS NORMAL AND OSTEOPOROTIC 24 HOUR URINE VALUES				INFUSED NORMAL AND OSTEOPOROTIC GROUPS 24 HOUR URINE VALUES							
					NORMAL 24 HOUR URINE VALUES				OSTEOPOROTIC 24 HOUR URINE VALUES			
	CALCIUM		CITRATE		CALCIUM		CITRATE		CALCIUM		CITRATE	
	NORMAL	POROTIC	NORMAL	POROTIC	CONTROL	INFUSION	CONTROL	INFUSION	CONTROL	INFUSION	CONTROL	INFUSION
Statistical values												
Normal	22		22		10		10		16		16	
t	0.4		1.11		3.86		0.14		4.9		0.14	
p	> 0.5		> 0.1		< 0.005		> 0.5		< 0.001		> 0.5	

Acute Changes in Calcium and Citrate Excretion, Infused Normal and Osteoporotic Groups

	Normal						*Osteoporotic*					
Hr. of collection	Calcium (9-10) (11-12)	Citrate (9-10) (11-12)	Calcium (9-10) (12-1)	Citrate (9-10) (12-1)	Calcium (9-10) (1-2)	Citrate (9-10) (1-2)	Calcium (8-9) (11-12)	Citrate (8-9) (11-12)	Calcium (8-9) (12-1)	Citrate (8-9) (12-1)	Calcium (8-9) (1-2)	Citrate (8-9) (1-2)
Statistical values												
Normal	10	10	10	10	10	10	10	10	10	10	10	10
t	4.3	0.76	6.35	0.56	8.5	0.1	7.7	0.86	9.6	0.107	8.2	0
p	< 0.005	> 0.4	< 0.001	> 0.5	< 0.001	> 0.5	< 0.001	> 0.4	< 0.001	> 0.5	< 0.001	> 0.5

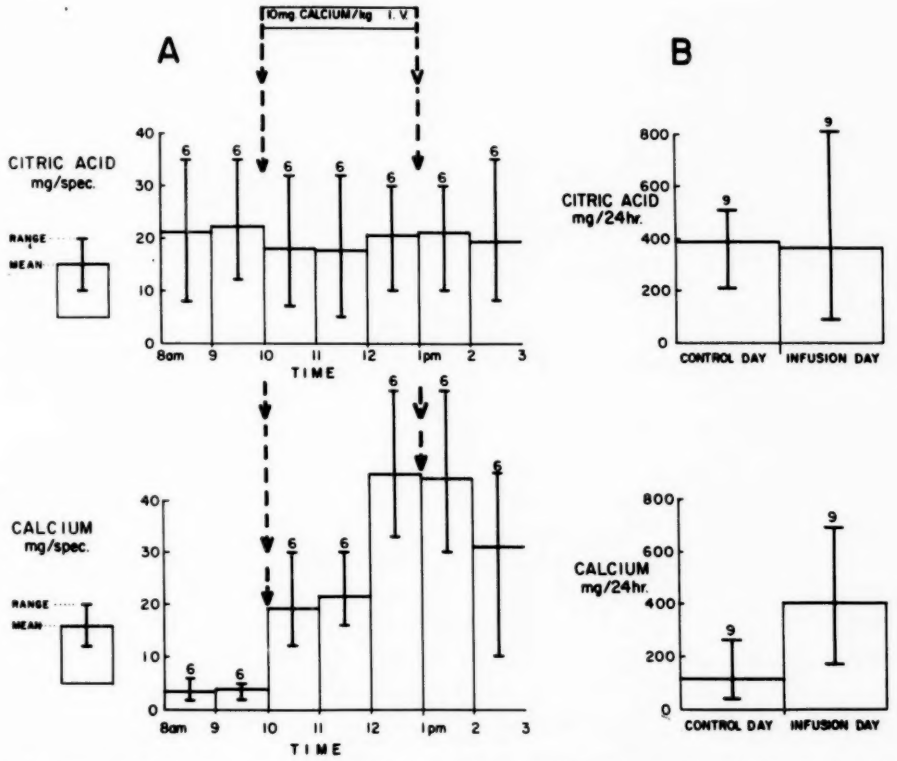

Fig. 3.—Changes in hourly and daily urine calcium and citric acid excretion induced by calcium infusion in osteoporotic patients.

and another normal man on constant calcium diets as subjects for the experiment. Urine was collected in 1 hour periods prior to and for 4 hours after the infusions. There was an immediate elevation of urine calcium excretion in both with parallel but small increments in citrate excretion in the first postinfusion hour; then despite continuing elevation of urine calcium levels, citrate excretion fell to levels below the control periods. A related experiment in this thesis was the measurement of urinary calcium and citrate levels in the author's urine collected in 3 hour fractions over a 24 hour period. In all but one period, citrate levels paralleled calcium excretion. It was concluded from these data that,

> In urine it is clear that the calcium content has a profound influence on the urinary citrate. Under ordinary physiological conditions any process altering the urinary calcium content, e.g. injection of calcium gluconate or parathyroid hormone was found to cause a parallel change in urinary citrate.

This conclusion has been reiterated more recently.[6]

On the basis of the data presented in this report, we are unable to detect any direct relationship between urinary calcium concentrations and citric acid excretion in normal or osteoporotic subjects. In both groups the changes observed in calcium and citric acid levels in the 24 hour collections and in the

shorter collections preceding, during, and after intravenous calcium gluconate demonstrated that the hypercalciuria produced by intravenous calcium gluconate in man had no clear-cut effect on citric acid excretion. While there seemed to be an apparent reduction in citrate excretion in the acute study, statistical evaluation revealed the changes to be insignificant. Chang and Freeman[10] found a marked reduction in urinary citrate in dogs after calcium chloride infusions. Milne[4] discounted their results and attributed them to the acidosis produced by the intravenous calcium chloride. His objection is not a completely valid one, however, since Chang and Freeman controlled this aspect of their experiment by infusing equivalent amounts of ammonium chloride, which increased urinary calcium concentration without effecting significant change in citric acid levels. Herrin and Lardinois,[11] studying citric acid clearances in the dog, found no change in citrate clearance during and after infusions of calcium gluconate and calcium lactate.

The data presented in this report point up another interesting dichotomy. There exists adequate evidence that calcium infusion causes an elevation in the plasma citrate level as does infusion of saline or potassium chloride.[15] Milne[5] has emphasized that the renal clearance of citrate is directly proportional to plasma citrate levels. The studies of Martenssen[16] on renal arteriovenous differences and of Chang and Freeman[10] which reveal a marked decline in urinary citric acid with a slight increase in blood levels of citrate in calcium chloride infused dogs, as well as data from the same laboratory on the differences in plasma citrate in nephrectomized and ureterally ligated dogs,[17] indicate that the kidneys play a significant role in the metabolic fate of citrate. Other reports indicate that renal tubular abnormalities of an idiopathic or acquired type may be associated with altered citrate excretion.[18-20] An increase in the blood level of citric acid, therefore, does not necessarily produce a concomitant change in its renal excretion. Plasma citrate levels were not followed in all the patients reported; however, when plasma citrate was measured, an elevation at the end of and after the calcium infusion occurred.

There is a paucity of data on urinary citric acid excretion in osteoporotic subjects. Edwards and co-workers[21] report that Estradiol Benzoate was without effect on urinary citrate levels in a postmenopausal woman and caused a delayed elevation of citric acid excretion in a girl with ovarian agenesis. Whedon and Shorr[9] found that some of the patients with poliomyelitis in their study developed roentgenographic evidence of osteoporosis of disuse without significant change in urinary citric acid output. The consistently lower mean citric acid excretion in the osteoporotic groups suggested that this might be a reflection of the basic bone disease. However, the differences from the normal group were not statistically significant.

These groups of patients were not matched as to age and this might be offered as another explanation for the difference in 24 hour excretion of citric acid between them. Since there was no statistically significant difference found between the groups in this regard we do not believe that this fact in any way alters our interpretation of the data.

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ABSORPTION OF Co^{60}-LABELED VITAMIN B_{12} IN MAN AND UPTAKE BY PARASITES, INCLUDING DIPHYLLOBOTHRIUM LATUM

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Intestinal absorption of vitamin B_{12} labeled with radiocobalt (Co^{60}), using the urinary excretion method of Schilling, was determined in six nonanemic patients infected with Diphyllobothrium latum, four with Taenia saginata, twelve with Ascaris lumbricoides, one with Necator americanus, one with Trichuris trichiura, and eight with Giardia lamblia. Normal urinary excretion ranging from 8 to 22 per cent of the oral dose was found in all instances, except that one patient with both giardiasis and sprue excreted only 1 per cent of the dose; this was thought to be caused by the sprue and not the giardiasis. Seven fish tapeworms (D. latum) showed significant uptake of Co^{60}-tagged vitamin B_{12}, varying from 2.6 to 13.7 per cent of the oral dose given to the host, whereas none of the other helminths showed any appreciable uptake. The relationship of these findings to the development of pernicious anemia caused by tapeworms and the geographic differences in the ability of D. latum to interfere with absorption of vitamin B_{12} and produce macrocytic anemia in the host are discussed.

THE purpose of this investigation was to determine whether certain parasitic infections alter the absorption of vitamin B_{12} in man and whether any significant amount of vitamin B_{12} is taken up by the parasites. A preliminary report has been submitted previously.[1] Infections with the following parasites were studied: *Diphyllobothrium latum, Taenia saginata, Ascaris lumbricoides, Necator americanus, Trichuris trichiura,* and *Giardia lamblia.*

D. latum was included because this parasite occasionally produces macrocytic anemia resembling pernicious anemia, especially in Finland[2, 3] and has been shown microbiologically to contain large amounts of vitamin B_{12}.[4] Previous studies by other investigators have shown impaired absorption of vitamin B_{12} labeled with radiocobalt (Co^{60}) in anemic patients harboring *D. latum* and significant uptake of tagged vitamin by this fish tapeworm.[5-10] Questions as to the exact mechanism of the ability of Finnish or northern European *D. latum* to produce these changes and whether *D. latum* from other parts of the world can demonstrate these phenomena as yet are not completely answered. Infection with *T. saginata* was used as a control, since this tapeworm has not

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been found microbiologically[4] to contain significant amounts of vitamin B_{12}, nor has it been demonstrated by radioisotopic studies[10] to alter absorption of the vitamin by the host; seldom, if ever, does it produce macrocytic anemia.[3]

A. lumbricoides was investigated because microbiologic assay[4] shows that this roundworm contains nearly as much vitamin B_{12} per unit weight as does *D. latum* but it rarely, if ever, produces macrocytic anemia.[3] *N. americanus* and *T. trichiura* were evaluated because of general interest and the occurrence of the latter worm in mixed infections in other patients studied; *G. lamblia* was studied because of the occasional association of this parasite with steatorrhea or a malabsorption syndrome.[11]

MATERIALS AND METHODS

Patients Studied.—Thirty-two patients harboring one or more of these parasites as identified by examination of stools were investigated. None of the patients were anemic and none had any significant associated diseases except for one with nontropical sprue. Some patients harboring one of the parasites being studied also had one or more of the following: *Enterobius vermicularis, Dientamoeba fragilis, Trichomonas hominis, T. trichiura, Amoeba coli, Endolimax nana, Hymenolepis nana,* or *Entamoeba histolytica.* Subsequent analysis of the data indicated that these mixed infections did not significantly alter the results.

Absorption of Vitamin B_{12}.—Intestinal absorption of vitamin B_{12} in the patients was measured by the urinary excretion method of Schilling,[12] as modified in our laboratories.[13, 14] The fasting patient is given by mouth a dose of about 0.6 mcg. of Co^{60}-labeled vitamin B_{12}* with a specific activity of about 800 μc per milligram, or 0.5 μc of Co^{60} in each dose. This is followed in 2 hours by a saturating dose of 1,000 mcg. of nonradioactive vitamin B_{12} injected parenterally. All urine is collected for the next 22 hours, and the radioactivity is measured using a scintillation counter (thallium activated, sodium iodide phosphor), compared with a duplicate of the dose, and corrected for background radioactivity. The results are expressed as a percentage of the test dose excreted in the urine. The normal range is 8 to 38 per cent. Approximately three times as much Co^{60}-labeled vitamin B_{12} is absorbed from the intestine as is excreted in the urine.

Expulsion of Parasites and Studies of Vitamin B_{12}.—One to 6 days after the oral administration of Co^{60}-labeled vitamin B_{12}, the helminths were expelled. For removing the various tapeworms, the anthelmintic used was an emulsion of oleoresin of aspidium; piperazine citrate or phosphate (Antepar) was used for the ascarides, hexylresorcinol for *N. americanus,* and dithiazanine iodide (Delvex) for *T. trichiura.* The parasites were recovered from the feces by use of the technique of Thompson,[15] using a 20 mesh sieve with a diameter of 12 cm. and with sufficient washing in warm water to remove debris. A careful search for parasites, especially the heads of tapeworms, was conducted after the contents of the sieve were emptied into a black enameled pan. Immediately on recovery, the worms were rinsed free of surface materials and checked for radioactivity by means of a scintillation counter. All specimens were checked in standard vessels with a constant volume, and both background activity and counts of an aliquot of the dose of the radioactive material were determined before and after measuring the radioactivity of the parasites. The results were expressed as the percentage of the oral dose given to the host patient that was taken up by the worm.

RESULTS

Absorption of Co^{60}-Labeled Vitamin B_{12} by Host.—A normal urinary excretion of Co^{60}-labeled vitamin B_{12} ranging from 8 to 22 per cent was found in

*The labeled vitamin B_{12} was obtained from Abbott Laboratories, North Chicago, Ill., under authorization of the Atomic Energy Commission.

all the types of parasitism studied except for the one patient who had giardiasis plus nontropical sprue, who excreted only 1 per cent of the test dose (Table I). This impaired absorption was not improved when the test was repeated, using 30 mg. of intrinsic factor orally with the Co^{60}-tagged vitamin B_{12}; this is the type of response seen in about 80 per cent of patients with nontropical sprue.[14, 16, 17] Therefore, it was concluded that the impaired absorption was caused by the nontropical sprue rather than the presence of *G. lamblia*.

TABLE I. ABSORPTION OF Co^{60}-LABELED VITAMIN B_{12} IN PARASITISM AS DETERMINED BY URINARY EXCRETION

PARASITE	PATIENTS	URINARY EXCRETION OF Co^{60}-B_{12} (% ORAL DOSE*)	
		AVERAGE	RANGE
D. latum	6	14.5	9-22
T. saginata	4	13.2	12-17
A. lumbricoides	12	15.4	8-22
N. americanus	1	22.0	-
T. trichiura	1	16.0	-
G. lamblia	7	16.6	10-22
Giardiasis + sprue	1	1.0	-

*Dose = 0.6 mcg.

Uptake of Co^{60}-Labeled Vitamin B_{12} by Parasites.—*D. latum* was the only one of the helminths studied that demonstrated any significant uptake of vitamin B_{12}, showing a mean uptake of 6.6 per cent in seven worms, with a range of 2.6 to 13.7 per cent (Table II). The 10 ascarides were recovered from six of the patients, with one to three worms being obtained from each host. Groups of these worms were tested as a single unit. The highest uptake (2.1 per cent) was found in two worms from one patient. Two tapeworms were expelled from one patient each with *D. latum* and *T. saginata* and were tested as single units. The uptake of 2.4 per cent was observed in a single *T. saginata*. The five specimens of *N. americanus* and the two of *T. trichiura* were obtained from a single but different host, and each group was tested as a single unit.

Studies on D. latum.—The results in the six nonanemic patients harboring *D. latum* are summarized in Table III. The ages of these patients varied widely; the sex distribution was equal. The tests were done 3 to 6 days after administration of the oral dose of Co^{60}-labeled vitamin B_{12} to the host, and there was little significant difference in the uptake by the worms, although the greatest uptake of 13.7 per cent was recorded in a worm that was in the intestine for 6 days after the oral dose before being expelled. A similar percentage was found in two worms expelled from one host after 4 days and tested as a single unit. The urinary excretion was normal in all patients. In four patients, the Schilling test was repeated a few days after the fish tapeworm was expelled. Normal urinary excretion was observed in three of these patients. In the fourth patient, the urinary excretion was slightly less than normal (6 per cent) and it increased to 9 per cent when the test was repeated with the addition of intrinsic factor. This patient had mild diarrhea at the time of these tests, which could have been a factor in the results.

TABLE II. UPTAKE OF Co^{60}-LABELED VITAMIN B_{12} BY INTESTINAL PARASITES

PARASITE	NO. OF WORMS TESTED	UPTAKE OF Co^{60}-B_{12} BY PARASITE (% ORAL DOSE* GIVEN TO HOST)	
		AVERAGE	RANGE
D. latum	7†	6.6	2.6-13.7
T. saginata	5†	0.5	0- 2.4
A. lumbricoides	10‡	0.5	0- 2.1
N. americanus	5§	0	0
T. trichiura	2†	0	0

*Dose = 0.6 mcg.
†Two worms expelled from one patient and tested as a unit.
‡Recovered from six patients and tested as six units.
§Obtained from one patient and tested as a unit.

Some observations were made relative to the site of uptake of Co^{60}-labeled vitamin B_{12} by the fish tapeworm. One of the worms recovered in case 4 (Table III) was counted, blotted dry, and placed on roentgenographic film between plates of glass in a darkroom and allowed to remain there for 10 months. The greatest concentration of radiation was in those segments or proglottids in the sections just distal to the scolex and neck of the worm, where growth activity is greatest, with less evidence of radiation being noted in sections in the middle or distal portion of the worm, where the proglottids are sexually mature or gravid.

TABLE III. ABSORPTION OF Co^{60}-LABELED VITAMIN B_{12} IN NONANEMIC PATIENTS AND UPTAKE BY *D. latum*

CASE	AGE (YR.)	SEX	DAYS AFTER DOSE TO RECOVERY OF WORM	% ORAL DOSE* IN WORM	URINARY EXCRETION BY PATIENT (% ORAL DOSE*)	
					WITH WORM	AFTER WORM EXPELLED
1†	52	M	3	2.6	16	-
2	36	F	3	5.4	15	6
3‡	18	M	3	7.0	14	-
4§	40	F	4	13.7	9	17
5	58	F	5	4.0	14	16
6	43	M	6	13.7	22	23
Average			4.2	6.6	14.8	15.5
Range			3-6	2.6-13.7	9-22	6-23

*Dose = 0.6 mcg.
†An extremely small worm, with many segments passed prior to test.
‡Head of worm not recovered.
§Two worms recovered and tested as a single unit.

Preliminary measurement of radioactivity in various sections of the worm with a scintillation counter also revealed greater concentrations in the proximal proglottids, where growth is active, than in the mature middle or distal proglottids.

COMMENT

The lack of demonstrable interference with the intestinal absorption of Co^{60}-labeled vitamin B_{12} in the presence of any of the parasites studied was anticipated. None of the patients were anemic, and only *D. latum* is known to be associated occasionally with macrocytic anemia. Klayman and Brandborg[5] likewise found no impairment of absorption of vitamin B_{12} in the presence

of *D. latum* in a single patient. Krevans and associates[16] noted impaired absorption in one patient convalescing from acute infectious hepatitis and harboring ascarides in the intestine, but hematologic abnormalities were absent. As already mentioned, the impaired absorption in our one patient with nontropical sprue plus giardiasis was thought to be due to the sprue.

None of the helminths tested showed any significant uptake of Co^{60}-labeled vitamin B_{12} except *D. latum*. The percentage of the dose taken up by these fish tapeworms (2.6 to 13.7 per cent) was about the same as that reported from Sweden[9] but was much less than that reported by Nyberg[10] from Finland (14.1 to 83.6 per cent) with a comparable test dose. Hookworms *(N. americanus)* and whipworms *(T. trichiura)* are small, few in number, and usually situated in the lower part of the small intestine or the upper part of the colon, so their lack of uptake was not surprising. However, study of hookworms and whipworms expelled from only one patient each is not sufficient to allow definite conclusions. Because the beef tapeworm, *T. saginata*, has been shown microbiologically to contain only extremely small amounts of vitamin B_{12}, a significant uptake was not anticipated. Nyberg's work[10] agrees with this. On the other hand, *A. lumbricoides* has a vitamin B_{12} activity as measured microbiologically almost as great as that of *D. latum*,[4] but none of the roundworms tested contained any significant amount of Co^{60}-tagged vitamin B_{12}. This parasite apparently does not compete actively with the host for its source of vitamin B_{12}.

A large proportion (20 to 25 per cent) of the Finnish population are carriers of *D. latum*, but only a small number (0.01 to 0.9 per cent) of these carriers have macrocytic anemia similar in characteristics to pernicious anemia.[3, 4] This anemia has been explained on the basis of a vitamin B_{12} deficiency.[2, 3] Patients who have anemia associated with tapeworms have low values of vitamin B_{12} in the serum[9, 18] and impaired vitamin B_{12} absorption.[6, 9] In nonanemic carriers, the absorption of vitamin B_{12} may be normal or slightly decreased. In both anemic and nonanemic patients, expulsion of the worm results in improved absorption of vitamin B_{12}. Forcing the tapeworm to a lower level in the intestine without destroying it results in increased absorption of vitamin B_{12} by the host.[3] Some investigators have found an improvement in absorption of radioactive vitamin B_{12} by the host and less uptake by the tapeworm in the presence of added intrinsic factor; therefore, they have suggested that competition between host and parasite occurs at the level of intrinsic factor of the host versus absorptive action of the worm.[6, 9] Patients with ordinary pernicious anemia also may harbor *D. latum*, but the absorption of vitamin B_{12} is not improved by expulsion of the worm.[8]

The uptake of vitamin B_{12} by *D. latum* is specifically metabolic, and the vitamin occurs in the bound form in the worm.[19] The fish tapeworm takes up significantly large amounts of vitamin B_{12} to limit the vitamin available for the host. The individual uptake varies greatly among worms tested in any given country. Furthermore, much larger amounts have been found in the tapeworms tested in Finland[7, 10] than in those reported from Sweden,[9] eastern

North America,[5] or in our own series.[1] Four of our patients were from south central Canada, and one each was from Minnesota and Iowa, with a history of visiting and eating fish in Canada.

It is interesting to speculate why *D. latum* in Finland or northern Europe takes up sufficient vitamin B_{12} that the host may exhibit macrocytic anemia, whereas this tapeworm rarely, if ever, produces such an anemia in other parts of the world. Perhaps there are different varieties or subspecies of *D. latum* with differing metabolic demands and host relationships. The amount of vitamin B_{12} in the diet and bodily stores may be important. Von Bonsdorff[3] suggested the following requirements for the development of pernicious anemia associated with the fish tapeworm: situation of the worm in the proximal part of the small intestine, poor dietary supply of vitamin B_{12}, reduced production of intrinsic factor by the host, and the presence of the worm for a sufficient length of time. In addition, the amount of vitamin B_{12} taken up by the tapeworm would be an important factor.

SUMMARY

The intestinal absorption of vitamin B_{12} labeled with radiocobalt (Co^{60}) was studied in a group of thirty-two patients infected with various intestinal parasites. None of the parasites studied *(Diphyllobothrium latum, Taenia saginata, Ascaris lumbricoides, Necator americanus, Trichuris trichiura,* and *Giardia lamblia)* interfered significantly with the intestinal absorption of Co^{60}-labeled vitamin B_{12} in the host, either directly or indirectly by excessive uptake of the vitamin.

Significant amounts of Co^{60}-tagged vitamin B_{12} were taken up by the fish tapeworm, *D. latum,* but not by any of the other helminths investigated.

Anemia did not occur in any of the patients harboring *D. latum* from North America, as compared to the occasional appearance of macrocytic anemia in carriers of *D. latum* in Finland.

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EFFECT OF CHLORAMPHENICOL ON ERYTHROPOIESIS

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Hematologic studies were done on twenty-two patients treated with chloramphenicol for various bacterial infections. In ten of these (receiving 40 to 85 mg. per kilogram per day for 4 to 27 days) multiple large vacuoles appeared in the primitive erythroblasts, often associated with a fall in red cell and reticulocyte counts. Where follow-up studies were obtained these changes had reverted to normal one week after chloramphenicol had been discontinued. In the other twelve patients (receiving 11 to 45 mg. per kilogram per day for 4 to 59 days) no such changes developed.

Four patients with pernicious anemia in relapse were given chloramphenicol (52 to 60 mg. per kilogram per day for 3 to 7 day periods) in addition to vitamin B_{12}. The marrows, though becoming normoblastic, showed vacuoles in early erythroblasts. Where administration of chloramphenicol was started 3 days prior to that of vitamin B_{12}, the characteristic reticulocyte response was delayed until after chloramphenicol had been discontinued. When administration of chloramphenicol was started 5 days after that of vitamin B_{12} had been started, there was an initial suboptimal reticulocyte response, then a rapid fall, and finally a second peak 8 days after the last dose of chloramphenicol. In two patients with iron deficiency given iron-dextran and chloramphenicol simultaneously, erythroblast vacuoles and interruption of reticulocyte response were also obtained.

Seven normal volunteers were given chloramphenicol (44 to 71 mg. per kilogram per day) for 7 days; one for an additional week, after two 500 ml. phlebotomies. There were no significant blood or marrow changes.

These findings suggest that chloramphenicol frequently causes reversible toxic degeneration of the erythroblasts and depression of reticulocytes. Increased dosage and accelerated erythropoiesis appear to favor these changes.

APLASTIC ANEMIA in patients treated with chloramphenicol has been reported repeatedly since 1950.[1-9] Most of the patients described had serious clinical manifestations and many died. The nitrobenzene moiety of the chloramphenicol molecule has been implicated as the responsible chemical factor but the exact mechanism of these untoward changes is unknown.[10]

The discovery of striking but reversible morphologic changes in the bone marrow associated with erythroid hypoplasia in patients treated with chloramphenicol led to this study of the effect of chloramphenicol on the blood and marrow in several clinical conditions. A preliminary report has been published.[11]

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CLINICAL MATERIAL

Group I.—Observations were made on twenty-two patients on chloramphenicol therapy. There were fifteen males and seven females, all but one were adults with an average age of 49 years. There were nine patients with pneumonia, eight with urinary tract infection, one each with osteomyelitis, pyarthrosis, peritonitis, wound infection, impetigo, and septicemia. The infecting organisms, where identified, included *Staphylococcus aureus, Bacterium aerogenes, Streptococcus pneumoniae, Pseudomonas aeruginosa, Klebsiella capsulatus,* and *Proteus ammoniae.* Sixteen of these patients received additional antibiotics.

Group II.—Chloramphenicol was given to six anemic subjects who were free from clinical infections; four patients had pernicious anemia in relapse and two had iron deficiency anemia. The diagnosis of pernicious anemia was made originally by the characteristic blood and marrow findings, and histamine-fast gastric achlorhydria; it was established eventually by return to normal hematologic values after vitamin B_{12} therapy. The diagnosis of iron deficiency anemia was based on the characteristic blood findings and an absence of marrow hemosiderin; it was established by a return to normal hematologic values after iron therapy.

Group III.—Chloramphenicol was given to seven healthy adult male volunteers with normal blood pictures.

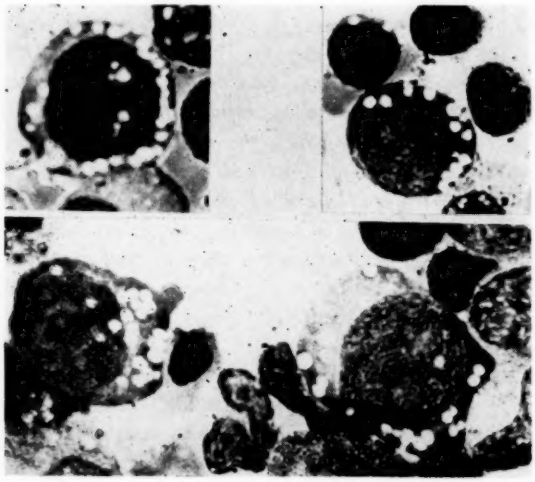


Fig. 1.—Vacuolated bone marrow erythroblasts. *A*, Patient A. W. (Group I); *B*, Patient A. H. (Group I); *C*, Patient M. C. (Group II; see also Fig. 6).

METHODS

Sternal bone marrow aspirations were obtained on the last day of chloramphenicol administration. Blood and marrow studies were performed by standard methods.[12] Reticulocytes were counted by the dry method using brilliant cresyl blue and prepared cover slips. Chloramphenicol* was given daily, orally, in four divided doses at 6 hour intervals. Dosage details are given later.

OBSERVATIONS

General.—

Bone marrow: The outstanding bone marrow finding was the appearance of numerous vacuoles in the cytoplasm and nuclei of the primitive erythroblasts

*Chloromycetin was supplied by Dr. James R. Fitzsimmons of Parke, Davis & Company.

(Fig. 1). The vacuoles measured approximately 1 μ in diameter and numbered from 3 to more than 20 per cell. The vacuoles could not be stained by Sudan III or periodic acid–Schiff stain. Similar vacuoles were seen occasionally in granulocytes and megakaryocytes, and frequently in plasma cells. A marked decrease in nucleated red cells, primarily of late erythroblasts and normoblasts, was a common finding.

Blood: Red blood cells, reticulocytes, white blood cells, and platelets were often decreased in number when the marrow abnormalities were first seen, but there were no significant morphologic abnormalities. Details are given below.

Group I.—Ten patients, whose daily chloramphenicol dose had ranged from 40 to 85 mg. per kilogram (1.5 to 4 Gm.) for 4 to 27 days, developed vacuolated

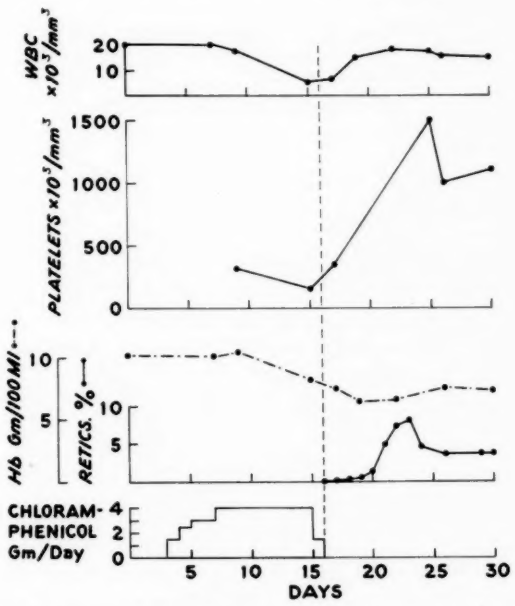


Fig. 2.—R. J. was a 36-year-old white man with *Staph. aureus* pneumonia and septicemia. Chloromycetin was discontinued after he had been afebrile for 8 days. A spontaneous reticulocyte peak occurred 8 days after chloramphenicol had been withdrawn. (See also Fig. 8.)

TABLE I. BONE MARROW ERYTHROID CELLS

PATIENT	DAYS ON CHLORAM-PHENICOL	TOXIC* CELLS /100 NR†	PRIMITIVE‡ CELLS /100 NR		NR/1,000 W.B.C.	
			DURING TREATMENT	OFF THERAPY§	DURING TREATMENT	OFF THERAPY
R. J.	14	47	49	2	73	222
A. W.	27	81	85	7	21	947
M. B.	9	37	40	6	75	663

Three patients with infections (Group I). R. J., see legend for Fig. 2. A. W. was an 18-year-old white woman with *Staph. aureus* pneumonia. She had been afebrile for 4 days when chloramphenicol was withdrawn. M. B. was a 40-year-old white woman with beta hemolytic streptococcus rectal abscess and diabetes.

*Toxic: Cells with multiple vacuoles in cytoplasm and/or nucleus.

†NR: Nucleated red cell.

‡Primitive cells: Proerythroblasts and early erythroblasts.

§Off therapy: Bone marrow obtained 1 week after chloramphenicol was discontinued.

erythroblasts. The other twelve patients, whose daily dose had ranged from 11 to 45 mg. per kilogram (1 to 3 Gm.) for 4 to 59 days, had normal appearing bone marrows at the end of the treatment. There were no significant differences in age, sex, type of infection, bacterial agents, or associated antibiotics between these two series of patients.

In some of the patients who developed vacuolated erythroblasts it was possible to make repeated hematologic observations after the discontinuation of the drug; Fig. 2 demonstrates the spontaneous increase in the number of reticulocytes, red cells, white cells, and platelets in one such patient. Seven days after the last dose of chloramphenicol, bone marrow erythroblast vacuoles had disappeared and nucleated red cells had greatly increased in number (see Table I).

F. C., a 27-year-old white man, developed the marrow and blood changes while receiving 71.5 mg. per kilogram body weight (3 Gm.) of chloramphenicol daily for 5 days for bacterial pneumonia. After recovery from pneumonia a repeat trial of 95 mg. per kilogram (4 Gm.) daily of the drug for 13 days failed to reproduce these changes.

Group II.—The six anemic patients received 3 to 4 Gm. of chloramphenicol daily. Erythroblast vacuoles appeared in all.

In the four patients with pernicious anemia, whose marrows were re-examined after vitamin B_{12} and chloramphenicol had been given, vacuoles had appeared in proerythroblasts, but megaloblasts had been replaced by the normoblastic maturation. The normal erythroid maturation occurred even though there had been only a suboptimal reticulocyte response (Figs. 3 and 4) or none at all (Fig. 5). In two patients there was a rapid decline from the initial reticulocyte response to vitamin B_{12} when chloramphenicol was added and a second reticulocyte response when chloramphenicol was discontinued (Figs. 3 and 4). In a third patient administration of chloramphenicol was started 3 days prior to vitamin B_{12} therapy. The expected reticulocyte rise failed to occur; when chloramphenicol was stopped on the seventh day there was a prompt reticulocytosis with a peak on the fifteenth day of vitamin B_{12} therapy (Fig. 5). The fourth patient with pernicious anemia received chloramphenicol only during the first 2 days of vitamin B_{12} therapy; vacuoles developed, but a normal reticulocyte response occurred.

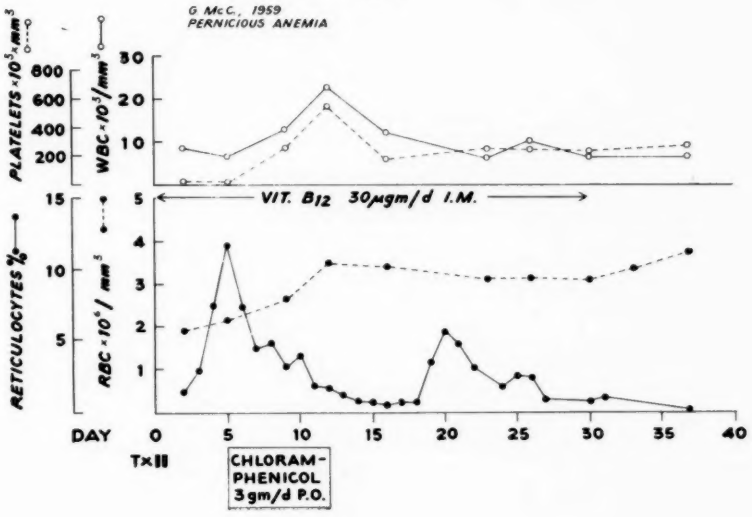
Chloramphenicol was given concomitantly with iron-dextran* to two patients with iron deficiency anemia. The reticulocytes, which were elevated prior to therapy, continued above normal for a few days, began to decline on the sixth day, and reached a nadir 10 days after chloramphenicol had been initiated. Secondary reticulocyte peaks occurred 8 days after discontinuation of chloramphenicol (Figs. 6 and 7). Patient M. C. reached a secondary reticulocyte peak of 26.5 per cent; this was associated with an outpouring of 35 nucleated red blood cells per 100 white blood cells, and leukocytosis of 41,000 per cubic millimeter.

*The iron-dextran referred to is Imferon, prepared by Benger Laboratories, Ltd., Holmes Chapel, Cheshire, England; it was obtained in this country from Lakeside Laboratories, Inc., Milwaukee, Wis.

Comparative reticulocyte responses in patients with pernicious anemia and patients with iron deficiency are given in Fig. 8. In every case the secondary peak occurred on the eighth day after the last dose of chloramphenicol.

Group III.—The normal volunteers were given 4 Gm. of chloramphenicol daily (44 to 71 mg. per kilogram) for 7 days. None developed blood or marrow changes. One subject was given a second course (4 Gm. per day for 7 days) of chloramphenicol immediately after two phlebotomies of 500 ml. each. Again there was no demonstrable qualitative or quantitative hematologic or marrow change.

Fig. 3.



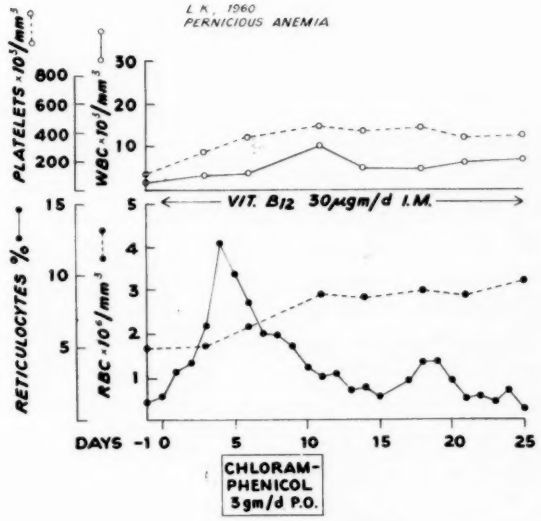


Fig. 4.

Figs. 3 and 4.—Effect of chloramphenicol on incipient remissions in pernicious anemia. Reticulocyte and red blood cell responses are interrupted and white blood cell and platelet rises are delayed.

DISCUSSION

Bone marrow depression may follow the administration of certain chemical and physical agents.[13] These potentially toxic substances may produce their effects in several different ways, e.g., they may alter the genetic nuclear material, inhibit mitosis, or impede nucleic acid synthesis, cell respiration, or hemoglobin synthesis. Host factors may be important in the development of these changes. There may be variation in the nutritional status or individual sensitivity; there may be differences in absorption, excretion, or enzymatic inactivation of the drug. Cardiovascular collapse in young infants treated with chloramphenicol presumably caused by the failure of glucuronide conjugation is an example of a host factor.[14-16]

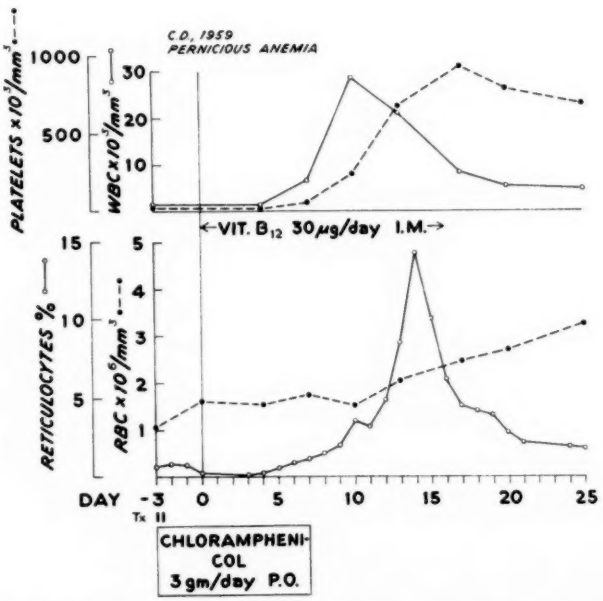


Fig. 5.—Preliminary chloramphenicol administration suppresses reticulocyte, red blood cell, white blood cell, and platelet response to vitamin B_{12} almost completely. When chloramphenicol is discontinued there is an exaggerated white blood cell and platelet response.

Reports of aplastic anemia caused by chloramphenicol are rare considering the widespread use of the drug.* Accounts of mild and reversible hematologic changes are even rarer.[17,18] Yet there is evidence that chloramphenicol frequently depresses erythropoiesis. Radioiron measurements in patients on chloramphenicol therapy showed delayed plasma clearance and diminished red cell uptake in five of fifteen subjects[19]; when large doses of chloramphenicol were given to four patients with cancer, all developed some degree of hematologic depression.[20]

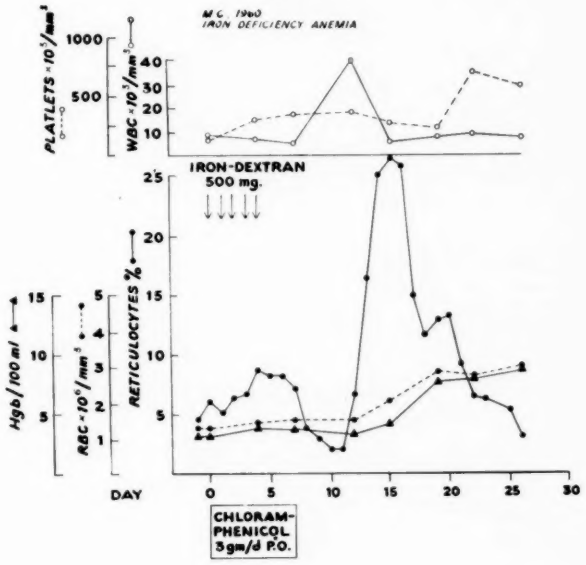
The data presented previously indicate that mild, reversible hematologic changes are not uncommon if carefully looked for. These changes may escape detection because anemia developing during chloramphenicol therapy might be

*Net sales 1958 in excess of $62,000,000; 1959 in excess of $70,000,000 (Parke, Davis & Company).

mistaken for anemia of infection, and a spontaneous rise in the red cell count and hemoglobin concentration, when the infection is controlled and chloramphenicol therapy is discontinued, seemingly would confirm this diagnosis.

The effect of chloramphenicol on marrow and blood could be studied well in anemic subjects. When chloramphenicol was administered, reticulocytes began to fall within 24 hours in two subjects with pernicious anemia on vitamin B_{12} therapy, but similar falls were delayed for approximately five days in two patients with iron deficiency anemia on iron-dextran therapy. In all

Fig. 6.



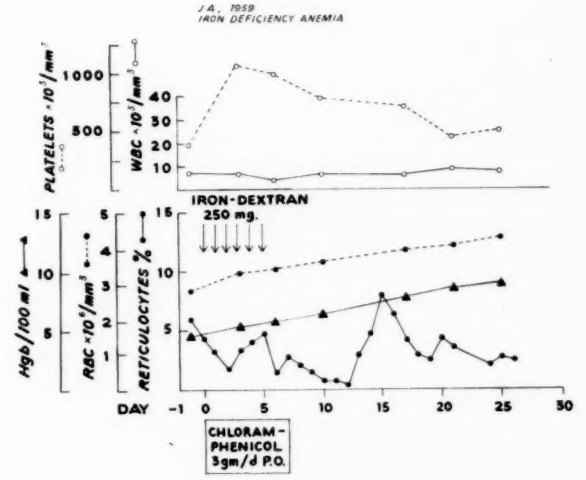


Fig. 7.

Figs. 6 and 7.—Effect of chloramphenicol on incipient remission in iron deficiency anemia. There is an abortive early reticulocyte rise. The absolute secondary reticulocyte peak in patient M. C. was 526,000 per cubic millimeter.

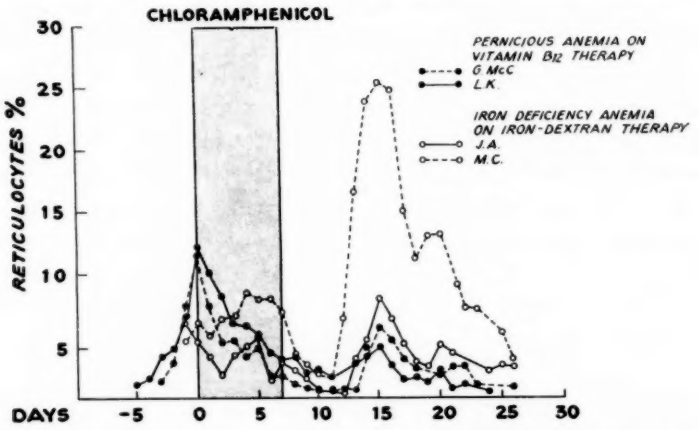


Fig. 8.—Comparative reticulocyte responses in four anemic patients receiving chloramphenicol in addition to specific antianemic therapy. The initial reticulocyte drop is faster in the patients with pernicious anemia, but in all patients the nadir is reached 10 to 11 days after the first dose of chloramphenicol. The secondary reticulocyte rise occurs in all patients 8 days after the chloramphenicol has been discontinued.

four of these patients reticulocytes were still moderately elevated between the fifth and tenth days but reached their lowest value 10 days after the first chloramphenicol dose. Since red blood cells retain their reticulum for only about 4 days, reticulocyte production and release must have continued for some time after the initial chloramphenicol dose. This 10 day lag between the initial and maximal reticulocyte depression in the patients with pernicious anemia and the delayed drop in patients with iron deficiency anemia imply that the major chloramphenicol marrow depression action is not directed against reticulocytes and late erythroblasts, but against younger cells. This interpretation is supported by the observation that vacuoles were not seen in late erythroblasts. The finding that in patients with pernicious anemia megaloblasts were replaced by normoblastic maturation, despite development of vacuoles in the erythroblasts, suggests that the action of chloramphenicol is not at stem cell level, but later, and does not interfere with purine and pyrimidine synthesis, the presumptive site of vitamin B_{12} action.[21] Chloramphenicol seems to act at the proerythroblast level, where a maximum vacuolization is seen. The relatively rapid return to high reticulocyte counts with consistent secondary peaks 8 days after the last dose of chloramphenicol is in favor of a block at an intermediary stage of development rather than destruction of the most primitive cells.

Most of the patients in this study received chloramphenicol for relatively short periods, usually 7 days. Aplastic anemia following chloramphenicol therapy has occurred in the majority of cases after repeated or prolonged therapy. It is therefore difficult to make comparisons and the events described here may be entirely unrelated to the pathogenesis of aplastic anemia. However, the severe reduction in the marrow nucleated red cells, most noticeable in patient A. W. (Group I) who also had the most marked vacuole formation, and who had been on chloramphenicol for 27 days, suggests that the difference may be only quantitative.

Although this study is primarily concerned with recording certain hematologic observations after chloramphenicol administration the data permit some tentative conclusions: (1) results in Group I (infections) suggest that dosage is a factor in the production of marrow changes; (2) the frequency of the hematologic changes observed in Groups I and II (anemias) cannot be explained by individual hypersensitivity alone; (3) the contrast between the high incidence of hematologic abnormalities in Groups I and II, and the absence of the changes in Group III (normal subjects) indicates the presence of host factors; (4) anemic subjects with active marrows appear to be particularly sensitive to the marrow depressing effects of chloramphenicol.

SUMMARY

1. Chloramphenicol was given to twenty-two patients with infections, to six anemic patients, and to seven normal individuals.

2. Ten of the patients with infections, all of the anemic subjects, but none of the normal individuals developed numerous vacuoles in the primitive bone marrow erythroblasts.

3. In the six anemic subjects, reticulocyte response to vitamin B_{12} or iron-dextran was interrupted or delayed by the administration of chloramphenicol.

4. Normal marrow and blood values became re-established when chloramphenicol was discontinued.

We are indebted to Mrs. Charlotte McMinn for the performance of the blood examination and to Mr. Marshall Douglas for the photomicrography.

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UNUSUAL RENAL FUNCTION ASSOCIATED WITH SALURESIS ACCOMPANYING WATER LOADING IN A CASE OF PRIMARY ALDOSTERONISM

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Renal function was investigated in a case of primary aldosteronism. Standard clearance methods were employed. Preoperatively glomerular filtration rate varied between 60 and 79 ml. per minute; renal plasma flow varied between 200 and 260 ml. per minute. After removal of an adenoma of the left adrenal cortex glomerular filtration rate and renal plasma flow declined to 20 and 80 ml. per minute, respectively. In addition to the previously reported urinary wasting of K^+ and HCO_3^- the patient demonstrated high phosphate clearance which varied between 20 and 40 ml. per minute. This phenomenon may be related to diversion of HCO_3^- from its major pathway of reasorption (ion exchange) to one in which it competes with phosphate for a transport system. After ingestion of a water load the subject excreted excessive quantities of Na^+ and Cl^- in the urine. This was found to be as high as 1mM. per minute whereas 24 hour sodium excretion was approximately 100 mM. and suggests that the adenoma was not behaving autonomously, but rather that its aldosterone secretion was responding to changes in the extracellular fluid volume. This fact may have important implications regarding the absence of edema in patients with primary aldosteronism.

RENAL dysfunction is a well-recognized part of the syndrome of primary aldosteronism and plays an important role in the pathologic physiology of this disorder. Both the associated systemic hypertension and potassium depletion are important factors contributing to the deterioration of renal function.

We wish to report detailed data on certain aspects of renal function in one patient and to emphasize its deterioration after removal of an adrenal cortical adenoma. We also wish to call attention to the heretofore unreported observation of marked saluresis associated with ingestion of large water loads and to speculate on its possible mechanism and implications.

CASE REPORT

The patient was a 59-year-old white man who was referred to the Minneapolis Veterans Hospital on May 25, 1959, because of persistent hypokalemia and systemic hypertension. He had no specific complaints. Hypertension was known to have existed since 1945. Between 1953 and 1959 he had received treatment with a variety of antihypertensive drugs.

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On January 15, 1959, treatment with 1 Gm. of chlorothiazide and 53 mEq. of potassium chloride per day was begun. On February 12, 1959, the serum potassium concentration was 2.9 mEq. per liter. Medication was stopped at that time. On April 23, 1959, he was started on 53 mEq. of potassium chloride because of persistent hypokalemia. After 21 days of treatment the serum potassium was 3.3 mEq. per liter. On admission to the hospital physical examination was not remarkable except for a blood pressure of 212/130 mm. Hg. Significant laboratory data revealed the serum electrolyte concentrations to be as follows: Na^+, 144 mEq. per liter; K^+, 3.4, 2.5, and 2.7 mEq. per liter; Cl^-, 104 mEq. per liter; inorganic phosphorus, 2.0 mg. per cent. The blood urea nitrogen was 23 mg. per cent. Whole blood arterial pH and urine pH were 7.64 and 7.46, respectively. Arterial blood plasma CO_2 content was 34.77 mM per liter. Total exchangeable K^+ and Na^+ were 27.4 and 49.5 mEq.

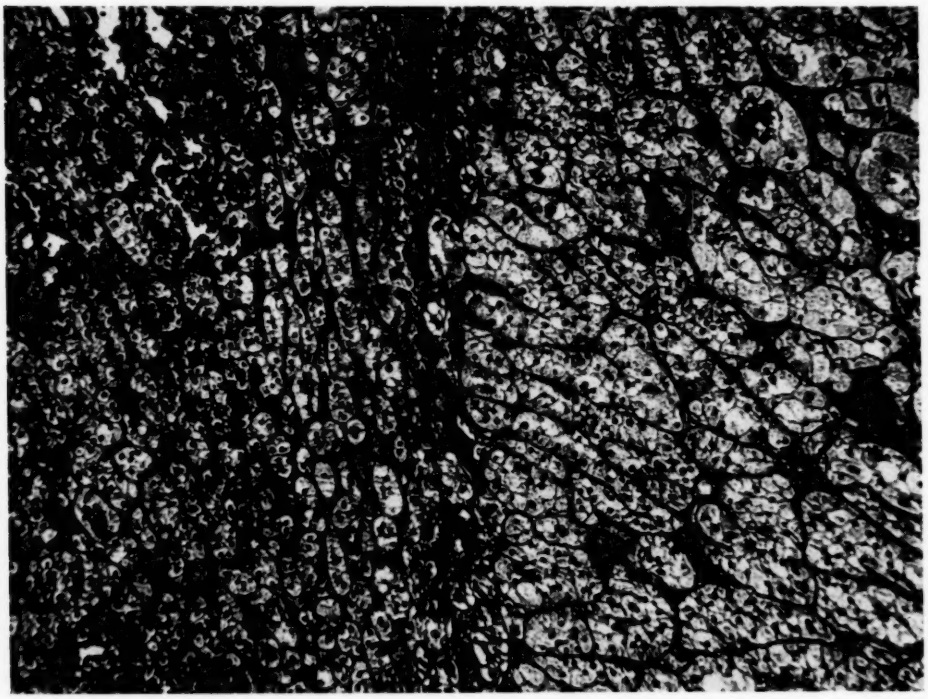

Fig. 1.—Section of the adrenal adenoma found in the left adrenal cortex. Grossly the tumor was bright yellow, well circumscribed, and measured 3 cm. in its largest diameter. The adenoma is on the right and is well demarcated from the hemorrhagic adrenal cortex to the left. The cells are large and vacuolated. Nuclear pleomorphism is prominent. (Hematoxylin and eosin. Magnification ×62; reduced ⅕.)

per kilogram body weight, respectively. Twenty-four hour urin aldosterone excreation was 52 μg per 24 hours (normal, 9 to 10 μg) as determined by the double isotope derivative assay technique* and varied from 50 to 162 μg per 24 hours (normal 10 to 35 μg) with the modified Neher-Wettstein method.† On July 23, 1959, an adenoma of the left adrenal cortex was removed (Fig. 1). The right adrenal gland was found to be normal. After the surgical operation the hypokalemic alkalosis disappeared and the blood pressure declined from preoperative values ranging between 200/110 and 220/120 mm. Hg to values ranging between 130/84 and 190/100 mm. Hg.

*Kindly performed by Dr. W. Moran, Department of Surgery, University of Minnesota Medical School.

†Kindly performed by Dr. T. Chao, Department of Medicine, Veterans Administration Hospital, Minneapolis, Minn.

METHODS

Clearance studies were performed during water diuresis with a catheter in the urinary bladder. All periods varied from 10 to 15 minutes in duration and were terminated by introduction of air into the bladder.* Urine samples analyzed for CO_2 content were collected anaerobically. Glomerular filtration rate (GFR) and renal plasma flow (ERPF) were estimated from the inulin and PAH clearances, respectively. Inulin was measured by the method of Roe and associates,[1] PAH by the method of Brun,[2] Na^+ and K^+ with a Baird flame photometer, Cl^- by the method of Schales and Schales,[3] and inorganic phosphorus by the method of Fiske and SubbaRow.[4] All pH determinations were carried out anaerobically at 37° C. in a Cambridge research model pH meter. Carbon dioxide determinations were done by the method of Van Slyke and Neill.[5] Osmolality determinations were made with the Fiske osmometer. Bicarbonate concentrations in both serum and urine were calculated as the difference between total CO_2 content and dissolved CO_2. Carbon dioxide tension in plasma and urine was calculated from the Henderson-Hasselbalch equation using a value of 6.1 for pK′ and values of 0.0301 and 0.0309 for 0.0591 α in blood and urine, respectively. Filtered load for all electrolytes was calculated as the product of the glomerular filtration rate and plasma or serum concentration of the ion in question without correction for the Donnan effect. Renal reabsorption was calculated as the difference between filtered load and urinary excretion. Total exchangeable K^+ and Na^+ were measured by the isotope dilution principle using K^{42} and Na^{22}. An equilibration period of 24 hours was used in both determinations.

RESULTS

Table I contains data obtained on four separate clearance studies. In the study of June 22, 1959, glomerular filtration rate was found somewhat reduced (average 75 ml. per minute). The patient exhibited a severe extracellular alkalosis (pH 7.64) and excreted a highly alkaline urine (pH 7.46) that contained excessive quantities of HCO_3^- (24.03 mM. per liter). The next study was performed after he had been on a diet containing 14 mEq. of Na^+ for 5 days. After two control periods (C) he received an intravenous infusion of 9.5 mg. of hexamethonium ion. This resulted in a drop in blood pressure from 210/120 to 124/80 mm. Hg. Clearance periods (E) were again begun after the blood pressure had stabilized at 150/90 mm. Hg. After this glomerular filtration rate dropped from 64 ml. per minute to approximately 39 ml. per minute. Renal plasma flow did not seem to drop correspondingly. The urine pH dropped to 5.67 and urine CO_2 concentration dropped to 4.95 mM. per liter. The study of July 22, 1959, was performed after 19 days of Na^+ restriction and 11 days of addition of 140 mEq. of K^+ to his diet. After three control periods (C) he received a rapid intravenous infusion of 3 per cent sodium chloride solution followed by a sustaining infusion during which three experimental periods (E) were obtained. This study shows the progressive decline in glomerular filtration rate and renal plasma flow. The extracellular alkalosis had been largely corrected (pH 7.47) but the urine remained alkaline (pH 7.08). In spite of a relatively normal blood pH the CO_2 concentration of plasma remained elevated (36.70 mM. per liter). Saline administration, if anything, produced a slight rise in blood and urine pH. It was without effect on K^+ excretion. One month postoperatively the glomerular filtration rate and

*In the study of July 8, 1959, clearance period E-2 was not started until a steady blood pressure had been maintained for 30 minutes after the intravenous administration of 9.5 mg. of hexamethonium ion.

renal plasma flow had declined still further to values of approximately 20 and 70 ml. per minute, respectively. The blood urea nitrogen thereafter ranged between 50 and 60 mg. per cent. The extracellular alkalosis disappeared postoperatively (pH 7.40). Table II illustrates the data on the renal handling of HCO_3^-. Normally practically all filtered HCO_3^- is reabsorbed below plasma levels of approximately 25 mM. per liter. Above this level HCO_3^- is excreted in the urine. In our laboratory normal HCO_3^- reabsorption is about 2.5 mM. per 100 ml. of glomerular filtrate. HCO_3^- reabsorption in this patient was greater than normal preoperatively with values ranging between 2.97 and 3.11 mM. per 100 ml. of glomerular filtrate. Table III shows the K^+ data. The severe degree of K^+ wastage is obvious especially on the studies of July 8, 1959, and July 22, 1959, when net secretion of K^+ may be seen in the last column. Table IV illustrates data on the renal handling of inorganic phosphorus. Preoperatively C_{PO_4} was supernormal (20 to 40 ml. per minute). With decline in glomerular filtration rate the per cent of filtered PO_4 reabsorbed declined. Yet C_{PO_4} remained high. After removal of the tumor C_{PO_4} dropped to a normal

TABLE I. RENAL HEMODYNAMICS, BLOOD AND URINE pH, PLASMA AND URINE CO_2 PLASMA AND URINE OSMOLALITY, AND URINE VOLUME IN FOUR STUDIES OF A PATIENT WITH PRIMARY ALDOSTERONISM

PERIOD	$C_{In.}$ (ML/MIN.)	C_{PAH} (ML/MIN.)	pH_B	pH_U	$[CO_2]_P$ (MM./L.)	$[CO_2]_U$ (MM./L.)	$[osm]_P$ (MOSM/Kg.H_2O)	$[osm]_U$ (MOSM/Kg.H_2O)	VOL. (ML./MIN.)
					6/22/59				
Blank							288	141	--
1	79.2		7.64	7.46	34.77	25.08	278	213	13.2
2	74.1							204	13.9
3	71.7						274	--	13.0
					7/8/59 (300 mg. Na diet, 5 days)				
Blank							--	83	--
C-1	63.7	259.6	7.60	7.00	34.77	20.24	274	245	8.9
C-2	65.0	256.6	7.53	7.24	35.10	18.04	273	213	12.0
					GFR ↓				
E-2	39.0	235.3	7.53	5.67	35.20	4.95	273	408	0.68
E-3							276	416	0.64
					7/22/59 (300 mg. Na. diet, 19 days)				
					(140 mEq. K added to diet, 11 days)				
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C-1	37.0	140.3	--	--	--	--	--	164	10.7
C-2	55.5	225.0	7.47	7.08	36.76	22.00	--	162	15.3
C-3	54.3	235.9	7.46	7.08	37.15	32.01	--	160	19.0
					3% NaCl intravenously				
E-1	53.1	200.1	7.48	7.18	39.75	21.01	--	236	18.2
E-2	64.3	256.1	7.50	7.17	35.27	20.90	--	236	19.0
E-3	60.0	212.7	7.51	7.20	35.20	23.10	--	221	16.6
					8/19/59 (1 month postoperatively)				
1	21.0	74.4	7.40	5.77	19.66	1.93			
2	19.8	67.1	7.40	5.78	19.86	2.70			
3	22.2	85.0	7.40	5.72	20.23	3.30			
4	24.1	67.0							
5	23.2	72.1							
6	23.4	68.9							

$C_{In.}$, inulin clearance; C_{PAH}, PAH clearance; pH_B, whole blood pH; pH_U, urine pH; $[CO_2]_P$, plasma CO_2 content; $[CO_2]_U$, urine CO_2 content; $[Osm]_P$, plasma osmolality; $[Osm]_U$, urine osmolality; Vol., urine volume. GFR↓ denotes the fall in inulin clearance produced by intravenous administration of 9.5 mg. of hexamethonium ion and is used in all subsequent tables.

value (12 to 14 ml. per minute) in spite of the presence of azotemia. Tables V and VI illustrate the Na^+ and Cl^- data. Preoperatively excretion of these ions was exceedingly high, amounting to over 1 mM. per minute in the initial study. Between 85 and 90 per cent of the filtered load of these two ions was reabsorbed in contrast to the 99 to 100 per cent observed normally. After the drop in glomerular filtration rate produced by hexamethonium reabsorption became complete. Postoperatively normal reabsorption of Na^+ and Cl^- was associated with marked impairment of glomerular filtration rate.

TABLE II. RENAL HANDLING OF BICARBONATE

PERIOD	LOAD (MM./MIN.)	EXCRETION (MM./MIN.)	REABSORPTION (MM./MIN.)	REABSORPTION (MM./100 ML. GFR)
		6/22/59		
1	2.64	0.32	2.32	2.97
		7/8/59 (300 mg. Na diet, 5 days)		
C-1	2.12	0.16	1.96	3.11
C-2	2.20	0.20	2.00	3.08
		GFR ↓		
E-2	1.32	--	1.32	3.38
		7/22/59 (300 mg. Na diet, 19 days) (140 mEq. K added to diet, 11 days)		
C-1	--	--	--	--
C-2	1.96	0.30	1.66	2.99
C-3	1.93	0.55	1.38	2.54
		3% NaCl intravenously		
E-1	1.77	0.35	1.42	2.67
E-2	2.18	0.37	1.81	2.81
E-3	2.03	0.36	1.67	2.78
		8/19/59 (1 month postoperatively)		
1	0.393	0.003	0.390	1.86
2	0.374	0.004	0.370	1.87
3	0.428	0.005	0.423	1.91

DISCUSSION

Disturbed renal hemodynamics in primary aldosteronism has been reported by numerous investigators. Chalmers and co-workers[6] reported a decline in inulin clearance from 49.2 ml. per minute preoperatively to 29 ml. per minute postoperatively whereas PAH clearance dropped from 267 to 156 ml. per minute. The postoperative blood urea nitrogen in their case was 68 mg. per cent. Mader and Iseri[7] reported a case with inulin clearance and PAH clearance of 55.5 and 445 ml. per minute, respectively. After surgical operation glomerular filtration rate was stated to have returned to normal in 3 months. Eales' and Linder's case[8] showed a decline in endogenous creatinine clearance from 109 to 78 ml. per minute postoperatively. Dustan and co-workers[9] reported 3 cases two of which showed significant declines in both mannitol clearance and PAH clearance postoperatively. Our patient showed a progressive decline in glomerular filtration rate and renal plasma flow. After surgical operation the glomerular filtration rate was approximately 20 ml. per minute whereas renal plasma flow was approximately 80 ml. per minute. These changes in renal hemodynamics are similar to those reported after the administration of

TABLE III. RENAL HANDLING OF POTASSIUM

PERIOD	LOAD (μM/MIN.)	EXCRETION (μM/MIN.)	% EXCRETED
		6/22/59	
1	169	150	88.7
2	154	138	89.6
3	153	129	84.3
		7/8/59 (300 mg. Na diet, 5 days)	
C-1	119.7	146.9	122.7
C-2	123.5	149.6	121.1
		GFR ↓	
E-2	69.4	40.8	58.8
		7/22/59 (300 mg. Na diet, 19 days) (140 mEq. K added to diet, 11 days)	
C-1	78.8	152	193
C-2	136.0	191	140
C-3	111.3	232	208
		3% NaCl intravenously	
E-1	118.4	163	138
E-2	133.7	219	164
E-3	139.8	192	137
		8/19/59 (1 month postoperatively)	
1	69.3	37.8	54.5
2	73.3	36.1	49.2
3	82.1	36.0	43.8
4	89.2	38.4	43.0
5	84.2	35.3	41.9
6	83.8	36.0	43.0

Numbers greater than 100 per cent in the last column are due to net secretion of K^+.

TABLE IV. RENAL HANDLING OF INORGANIC PHOSPHORUS

PERIOD	LOAD (μM/MIN.)	EXCRETION (μM/MIN.)	REABSORPTION (μM/MIN.)	% REABSORBED	C_{PO_4} (ML./MIN.)
			6/22/59		
1	51.72	14.22	37.50	72.5	21.8
2	44.46	14.08	30.38	68.3	23.5
3	43.52	14.52	29.00	66.6	23.9
			7/8/59 (300 mg. Na diet, 5 days)		
C-1	38.22	16.02	22.20	58.1	26.7
C-2	40.73	16.80	23.93	58.8	26.8
			GFR ↓		
E-2	30.68	6.83	23.85	77.7	8.7
			7/22/59 (300 mg. Na diet, 19 days) (140 mEq. K added to diet, 11 days)		
C-1	31.10	20.50	10.60	34.1	24.4
C-2	51.10	29.20	21.90	42.9	31.7
C-3	49.20	35.50	13.70	27.8	39.2
			3% NaCl intravenously		
E-1	47.40	34.90	12.50	26.4	39.1
E-2	58.30	36.70	21.60	37.0	40.5
E-3	55.60	31.40	24.20	43.5	33.9
			8/19/59 (1 month postoperatively)		
1	21.50	13.10	8.40	39.1	12.8
2	20.50	12.40	8.10	39.5	12.0
3	21.80	13.30	8.50	39.0	13.5
4	23.10	12.60	10.50	45.5	13.1
5	22.30	13.30	9.00	40.4	13.9
6	22.20	12.20	10.00	45.0	12.9

TABLE V. RENAL HANDLING OF SODIUM

PERIOD	LOAD (MM./MIN.)	EXCRETION (MM./MIN.)	REABSORPTION (MM./MIN.)	% REABSORBED
		6/22/59		
1	11.25	1.09	10.16	90.3
2	10.48	1.15	9.33	89.0
3	10.18	1.02	9.16	90.0
		7/8/59 (300 mg. Na diet, 5 days)		
C-1	9.22	0.83	8.39	91.0
C-2	9.28	1.02	8.26	89.0
		GFR ↓		
E-2	5.57	0.02	5.55	99.6
		7/22/59 (300 mg. Na diet, 19 days) (140 mEq. K added to diet, 11 days)		
C-1	4.97	0.61	4.36	87.7
C-2	7.33	0.88	6.45	88.0
C-3	7.49	1.07	6.42	85.7
		3% NaCl intravenously		
E-1	7.27	1.95	5.32	73.2
E-2	8.82	1.90	6.92	78.5
E-3	8.22	1.59	6.63	80.7
		8/19/59 (1 month postoperatively)		
1	2.65	0.02	2.63	99.4
2	2.50	0.02	2.48	99.4
3	2.80	0.02	2.78	99.5
4	3.04	0.02	3.02	99.3
5	2.92	0.02	2.90	99.3
6	2.95	0.02	2.93	99.2

TABLE VI. RENAL HANDLING OF CHLORIDE

PERIOD	LOAD (MM./MIN.)	EXCRETION (MM./MIN.)	REABSORPTION (MM./MIN.)	% REABSORBED
		6/22/59		
1	7.49	1.03	6.46	86.2
2	7.26	1.07	6.19	85.3
3	--	--	--	--
		7/8/59 (300 mg. Na diet, 5 days)		
C-1	5.97	0.73	5.24	87.8
C-2	6.15	0.90	5.25	85.4
		GFR ↓		
E-2	3.67	0.03	3.64	99.2
		7/22/59 (300 mg. Na diet, 19 days) (140 mEq. K added to diet, 11 days)		
C-1	3.35	0.47	2.88	86.0
C-2	5.00	0.69	4.31	86.2
C-3	5.01	0.81	4.20	83.8
		3% NaCl intravenously		
E-1	5.63	1.60	4.03	71.6
E-2	--	1.67	--	--
E-3	6.40	1.33	5.07	79.2
		8/19/59 (1 month postoperatively)		
1	2.08	0.12	1.96	94.2
2	1.97	0.12	1.85	93.9
3	2.21	0.13	2.08	94.1
4	2.41	0.19	2.22	92.1
5	2.32	0.19	2.13	91.8
6	2.32	0.21	2.11	90.9

hexamethonium in hypertensive[10] patients and also after adrenalectomy in the treatment of essential hypertension.[11] Reference to Figs. 2 and 3 reveals that the histologic picture found in renal biopsy sections from our patient is that of hypertensive nephropathy. One may assume that the decrease in perfusion pressure is not accompanied by a corresponding drop in renal vascular resistance.

Enhancement of HCO_3^- reabsorption was demonstrated repeatedly in our patient. It has been reported that K^+ depletion causes enhancement of renal HCO_3^- reabsorption.[12] Total exchangeable K^+ in our patient was 27.4 mEq. per kilogram of body weight. The mean normal value reported in his age range is 39.1 mEq. per kilogram of body weight.[13] K^+ deficiency may have contributed to the increase in HCO_3^- reabsorption; however, decreased glomerular filtration

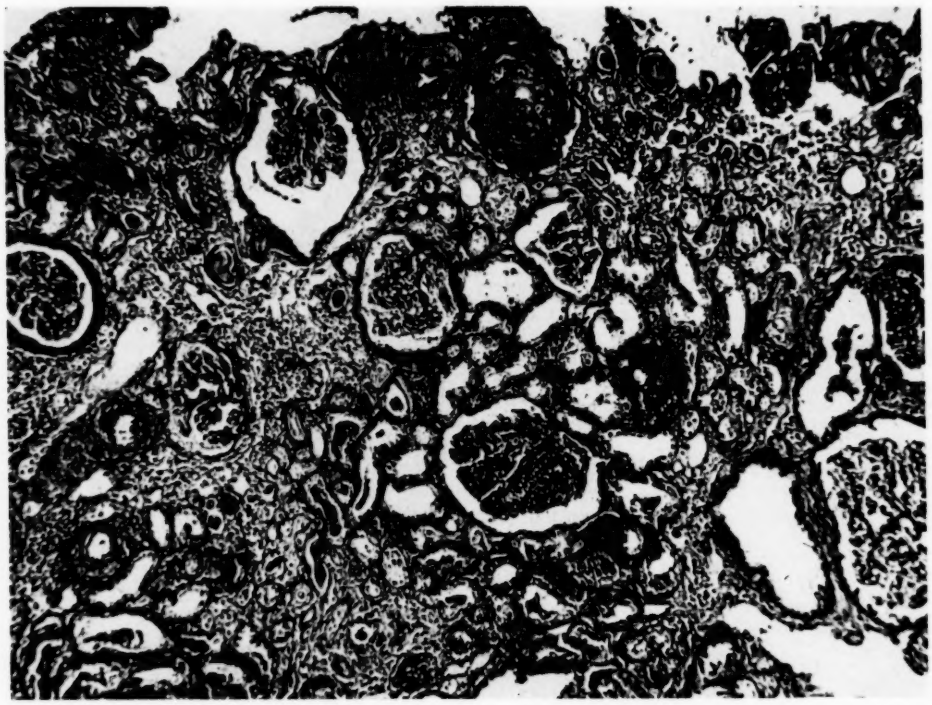

Fig. 2.—Section from a needle biopsy of the right kidney. There is widespread tubular atrophy and glomerular degeneration. Atherosclerosis of small arteries and arterioles is present. (Hematoxylin and eosin. Magnification ×62; reduced 1/5.)

rate is also known to produce increased $T_{M_{HCO_3^-}}$.[14] This phenomenon is well illustrated in Table II in the study of July 8, 1959. After the decrease in glomerular filtration rate produced by hexamethonium HCO_3^- reabsorption increased from 3.08 to 3.38 mM. per 100 ml. of glomerular filtrate. Probably part of the enhanced HCO_3^- reabsorption was the consequence of reduced glomerular filtration rate which was observed repeatedly. Therefore the data do not permit separation of the effects produced by K^+ depletion and decreased

glomerular filtration rate. Postoperatively the figures for HCO_3^- reabsorption are not T_M values since plasma HCO_3^- concentration had dropped to approximately 20 mM. per liter and the urine had become free of HCO_3^-.

The renal handling of inorganic phosphate was unusual. There was a persistent hypophosphatemia associated with high C_{PO_4} in the presence of low glomerular filtration rate. Mahler and Stanbury[15] reported hypophosphatemia and increased 24 hour C_{PO_4} in a case of "potassum-losing renal disease." They related the increased C_{PO_4} to the K^+ depletion pointing out that after therapy the plasma inorganic phosphorus rose to normal levels and the 24 hour C_{PO_4} declined to values ranging between 8 and 14 ml. per minute. Our data reveal that after the addition of 140 mEq. of K^+ to the diet the serum K^+ concentration rose from 2.7 to 5.3 mEq. per liter while the C_{PO_4} remained at values between 24 and 40 ml. per minute. Malvin and Lotspeich[16] reported a decrease in $T_{M_{PO_4}}$

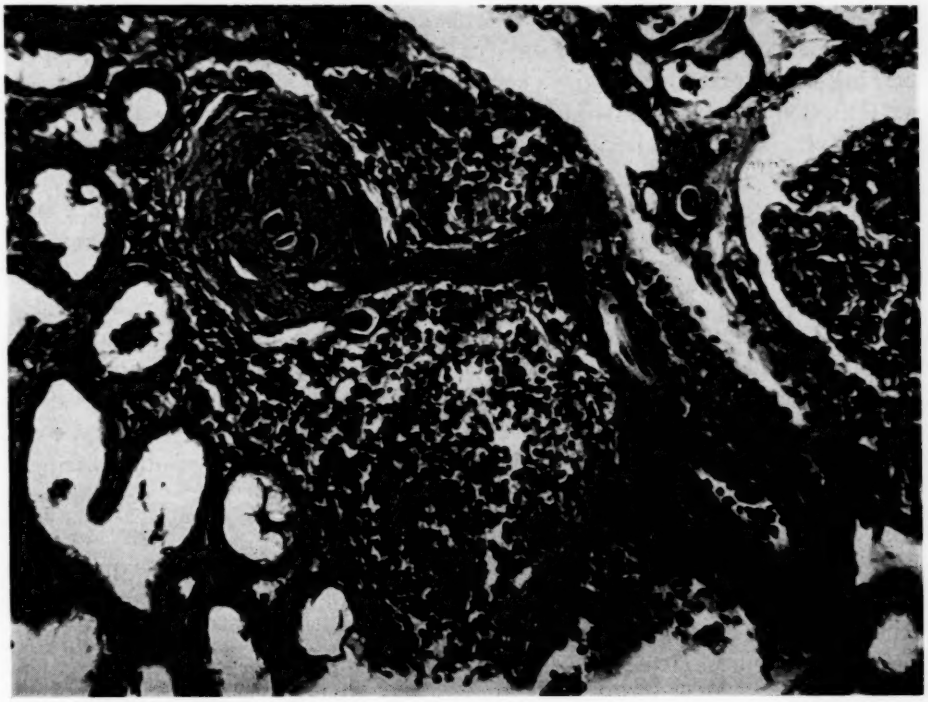

Fig. 3.—Section from a needle biopsy of the right kidney showing glomerular degeneration and arteriolar nephrosclerosis. (Hematoxylin and eosin. Magnification ×150; reduced ⅓.)

in dogs with procedures that increased HCO_3^- excretion. We have demonstrated a phosphate diuresis in human beings during bicarbonate diuresis produced by intravenous infusions of large doses of acetazolamide.[17] The present case may represent a similar phenomenon. The large quantities of HCO_3^- excreted by this patient suggest that the excess presented to the tubules for reabsorption may have competed with phosphate for a transport system resulting in diminished phosphate reabsorption.

An interesting problem is seen in the data for Na^+ and Cl^- excretion. This discussion will be limited to the case for Na^+ since the Cl^- data are similar. It will be noted in Table V that during the preoperative clearance studies Na^+ excretion was very high. In the study of June 22, 1959, it was above 1 mM. per minute. Such an excretion rate projected over a 24 hour period would result in approximately 1,400 mM. of urinary Na^+ per day! Measurement of 24 hour Na^+ excretion yielded values of approximately 100 mM. per day. For some reason this patient exhibited a severe Na^+ diuresis during the clearance studies. Corroboration of the data is seen in Table I which shows the urine osmolalities and volumes in the last two columns. The samples labeled blank were obtained prior to commencement of the studies and reveal osmolalities characteristic of those seen in water diuresis. Urines obtained during the clearance periods all showed osmolalities well above the blank samples. Furthermore, these osmolality values agree reasonably well with the sum of the determined urinary ions in the same samples. In all studies water loading of the patient was begun at 5:30 A.M., and clearance studies were started at approximately 8:00 A.M. The patient ingested 1,500 to 2,000 ml. of water in a period of 45 minutes after which he drank 250 ml. every half hour. This suggested to us that the large volume of water ingested may have been related to the NaCl diuresis. Dustan and associates[9] also observed excessive Na^+ excretion, but their patients were studied with osmotic diuresis which is known to reduce both Na^+ and Cl^- reabsorption.[18] Elkinton and coauthors[19] reported increased Na^+ excretion after intravenous infusion of salt-poor albumin. Orloff and co-workers[20] noted increases in Na^+ and Cl^- excretion after infusion of salt-poor albumin in nephrosis and in toxemia of pregnancy. Stewart and Rourke[21] infused large volumes of isotonic glucose and observed a fall in plasma Na^+ concentration and failure of the kidneys to conserve Na^+. Strauss and associates[22] observed an increased Na^+ excretion in water loaded subjects after intravenous infusions of a hypotonic electrolyte solution. The magnitude of Na^+ excretion in the studies cited was not as great as in our case.

Dehydration is known to prevent the increase in Na^+ excretion after administration of antidiuretic hormone (ADH).[23] Furthermore, expansion of the body fluids by the simultaneous administration of water and antidiuretic hormone results in reduced urinary aldosterone excretion.[24] Ingested water is known to distribute itself throughout the total body water rapidly.[25] This suggests that the large amount of water ingested by our patient prior to the studies resulted in expansion of the extracellular space sufficient in amount to decrease aldosterone secretion by the adrenal gland and that this resulted in the observed Na^+ diuresis. It is also possible that the ingestion of water directly altered the ability of the renal tubules to reabsorb Na^+, but there is no evidence to support this thesis. The implication is that the adrenal adenoma was responsive to a normal physiologic stimulus. It is possible that the remaining adrenal tissue responded to the increase in extracellular space with decreased aldosterone secretion. However, this is unlikely since K^+ depletion is known to decrease urinary aldosterone output[26, 27]; therefore we may assume that the

major site of aldosterone production in our patient was the adenoma. The recent report of Baulieu and co-workers[28] demonstrating decreased aldosterone excretion in a patient with primary aldosteronism after administration of 9α-fluoro-Δ'-cortisol with return to previous levels after cessation of the drug lends support to the concept that such adenomas may not behave autonomously.

Our observations may have important implications regarding the absence of edema in the vast majority of patients with primary aldosteronism. Such patients are known to exhibit polyuria which is usually attributed to K^+ depletion. However, it is possible that increased water intake may be of primary importance as a factor causing escape from the salt retaining effects of aldosterone in these patients and indeed in normal subjects to whom aldosterone[29] or DCA[30] is administered. In the latter groups the phenomenon is more difficult to explain on this basis since the action of exogenously administered compounds is presumably unaffected by changes in extracellular fluid volume.

SUMMARY

Renal function data are reported in a case of primary aldosteronism. After removal of a cortical adenoma of the left adrenal gland glomerular filtration rate and effective renal plasma flow declined to 20 and 80 ml. per minute, respectively.

Renal wasting of potassium, bicarbnate, and phosphate was corrected by removal of the tumor.

Marked saluresis was observed in association with ingestion of large water loads which suggested that aldosterone secretion by the adrenal adenoma was decreased after extracellular fluid space expansion.

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COMPARISON OF PULMONARY DIFFUSING CAPACITY IN NORMAL SUBJECTS AND IN PATIENTS WITH INTRACARDIAC SEPTAL DEFECTS

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Extensive pulmonary function tests have been done in ten normal individuals, nine patients with atrial septal defect, and eleven patients with ventricular septal defect. The results are similar in normal subjects and in patients except that pulmonary diffusing capacity is significantly increased in patients with septal defects, particularly those with atrial septal defects. The increased pulmonary diffusing capacity probably represents an increased size of the resting capillary bed necessary to accommodate the large pulmonary blood flow.

THIS study was undertaken to discover what functional changes occur in the lungs of patients who have intracardiac septal defects. Patients with normal pulmonary arterial pressure were chosen deliberately to avoid including patients with extensive pulmonary vascular disease. The diffusing capacity of the lungs (D_{LCO}) is dependent on the total capillary surface available for and actively involved in diffusion, the character and thickness of the alveolar capillary membrane, and the reaction rate of carbon monoxide with hemoglobin. Normal subjects and patients with septal defects should be similar with respect to the thickness and character of the alveolar capillary membrane and the reaction rate of carbon monoxide with hemoglobin. There might be more surface area actively involved in diffusion in the patients with septal defects because of the increased pulmonary blood flow. This should be particularly characteristic of the patients with atrial septal defect because these patients can have greatly increased pulmonary blood flow in the presence of normal pulmonary arterial pressure, whereas patients with ventricular septal defect and normal pulmonary arterial pressure have only mild to moderate increase in pulmonary blood flow.

MATERIALS AND METHODS

The normal persons were young men 17 to 24 years of age. The patients were men and women 14 to 42 years of age who had left-to-right shunts through atrial or ventricular septal defects. Each patient had a complete history, physical examination, roentgenogram of the chest, and necessary laboratory tests. The clinical diagnosis of atrial or ventricular septal defect was confirmed by cardiac catheterization in each patient and in most instances at operation also.

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TABLE I. PHYSICAL CHARACTERISTICS AND LUNG VOLUMES IN NORMAL SUBJECTS

PATIENT	AGE (YR.)	SEX	HEIGHT (IN.)	WEIGHT (POUNDS)	SURFACE AREA ($M.^2$)	LUNG VOLUMES						RV/TLC† RATIO × 100
						VITAL CAPACITY		RESIDUAL VOLUME		TOTAL LUNG CAPACITY		
						ML.	%*	ML.	%*	ML.	%*	
R.C.	17	M	73	164	1.94	5,620	116	1,240	102	7,025	123	19
W.C.	24	M	72	149	1.87	4,416	95	1,818	158	6,234	106	29
J.K.	21	M	70	159	1.88	4,582	98	1,847	157	6,429	110	29
J.M.	22	M	68	135	1.70	4,414	104	1,467	134	5,881	111	25
R.R.	23	M	67	170	1.87	4,480	96	1,175	102	5,655	106	19
L.S.	23	M	69	174	1.94	5,030	109	1,835	153	6,865	113	27
R.S.	24	M	69	175	1.95	5,080	104	1,230	100	6,310	103	18
B.S.	23	M	66	154	1.77	4,970	112	1,437	130	6,407	116	22
J.S.	23	M	73	198	2.14	5,460	102	1,780	134	7,240	110	24
H.W.	21	M	70	165	1.90	4,756	100	1,453	122	6,209	105	23
Mean	22		70	164	1.90		104		129		110	24

*Per cent of predicted value based on age and surface area.
†Residual volume/total lung capacity ratio × 100.

Pulmonary function tests were done in the morning after breakfast. Vital capacity, inspiratory capacity, and expiratory reserve volume were measured separately using a Benedict Roth spirometer. The maximum of at least three trials was recorded. The functional residual capacity of the lungs was measured in duplicate by the nitrogen washout method of Darling and collaborators.[2] Volumes were corrected to body temperature, ambient pressure, and saturated with water vapor (B.T.P.S.). Predicted normal values for vital capacity are calculated on the basis of the patient's surface area.[3] Predicted normal values for inspiratory capacity are 75 per cent of predicted vital capacity; predicted normal values for expiratory reserve volume are 25 per cent of predicted vital capacity; and predicted normal values for total lung capacity are predicted vital capacity divided by 0.80.[4] Rate, depth, and minute volume of ventilation were measured while the patient was breathing air, 99.6 per cent oxygen, and 7.5 per cent carbon dioxide in air. Measures of ventilation were made with the subject breathing through a mouthpiece. Oxygen and 7.5 per cent carbon dioxide in air were supplied from a tank by a demand valve regulator. Expired volumes were collected in a Douglas bag or a Tissot spirometer while arterial blood was being sampled. Volumes were corrected to body temperature, ambient pressure, and saturated with water vapor. Physiologic dead space was calculated using Bohr's equation. Measured and derived values used in this calculation are the volume of expired air over a 3 minute period, the per cent CO_2 in expired air, and the arterial P_{CO_2}. Calculation is based on the formula given by Comroe and associates.[5] Uniformity of intrapulmonary distribution of inspired gas was checked by the single breath nitrogen test of Comroe and Fowler[6] and the measurement of the percentage of nitrogen at the end of 7 minutes' breathing of oxygen as described by Cournand and coauthors.[7] To measure maximal breathing capacity (MBC), the subject breathed maximally for 15 seconds through a mouthpiece, low resistance valve, and wide tubing into a Tissot spirometer. Predicted normal values for the maximal breathing capacity are based on age and body surface area.[8] The highest value obtained with three trials was calculated in liters per minute. Maximal inspiratory (or expiratory) flow rate was measured between 200 and 1,200 ml. of the inspiration (or expiration) by the method of Danzig and Comroe.[9] The diffusing capacity of the lung for carbon monoxide ($D_{L_{CO}}$) was studied using the single breath method of Ogilvie and co-workers.[10] Predicted normal values for diffusion are based on body surface area.[10] The same formula is used for men and women.

In the patients, arterial blood was obtained from the brachial artery while the semirecumbent subject breathed air and again after he had breathed 99.6 per cent oxygen for at least 10 minutes. The samples were analyzed for total oxygen content, oxygen capacity, and whole blood carbon dioxide content by the manometric technique of Van Slyke and Neill.[11] Blood was rotated with air in a tonometer at room temperature for 20 minutes before measuring oxygen capacity. Appropriate corrections were made for physically dissolved oxygen and for differences in total hemoglobin content in the capacity samples. Direct measurement of dissolved oxygen was not made, but an approximate calculation was made on the samples collected during the inhalation of oxygen by subtracting the oxygen capacity from the total oxygen content. Arterial blood pH was measured in a closed Cambridge glass electrode at 37° C. unless done at room temperature when the pH value was corrected to 37° C. using Rosenthal's factor.[12] Plasma carbon dioxide content and P_{CO_2} were determined from pH, hematocrit, oxygen saturation, and whole blood carbon dioxide content using the nomogram of Singer and Hastings.[13]

Cardiac catheterization was done in the early morning with the patient fasting and resting in a supine position. Phasic arterial pressure was measured with a capacitance manometer and registered on a multi-channel direct writing oscillograph (Sanborn). Systemic blood flow and pulmonary blood flow were estimated using the Fick equation. Oxygen consumption was measured by collecting the patient's expired air in a calibrated Tissot-type spirometer and measuring partial pressure of oxygen in this sample and in room air with a Beckman model C oxygen analyzer, or it was estimated by using a value of 200 ml.

TABLE II. PHYSICAL CHARACTERISTICS AND LUNG VOLUMES IN PATIENTS WITH INTRACARDIAC SHUNTS

PATIENT	CLINICAL DIAGNOSIS	AGE (YR.)	SEX	HEIGHT (IN.)	WEIGHT (POUNDS)	SURFACE AREA (M.²)	LUNG VOLUMES										RV/TLC† X 100
							INSPIRATORY CAPACITY		EXPIRATORY RESERVE VOLUME		VITAL CAPACITY		RESIDUAL VOLUME		TOTAL LUNG CAPACITY		
							ML.	%*	ML.	%*	ML.	%*	ML.	%*	ML.	%*	
M.C.	ASD	21	M	71	144	1.82	3,100	91	2,060	181	5,160	113	2,800	246	7,960	140	35
M.Mc.	ASD	24	F	64	124	1.58	1,880	79	1,110	141	2,990	95	700	87	3,690	93	19
B.P.	ASD	30	F	65	114	1.56	2,210	94	930	119	3,140	101	2,060	264	5,200	133	40
D.P.	ASD	17	M	76	181	2.10	3,650	93	2,420	185	5,400	103	1,410	108	6,810	104	21
E.R.	ASD	42	M	69	129	1.70	2,450	77	1,620	153	4,070	96	3,170	244	7,240	130	44
S.D.	ASD	14	F	64	109	1.50	1,690	75	940	125	2,630	88	1,170	156	3,800	101	31
B.M.	ASD	16	F	61	116	1.49	1,960	88	890	119	2,850	96	950	127	3,800	102	25
G.V.	ASD	19	F	62	100	1.42	1,940	91	1,170	165	3,110	110	2,400	338	5,510	155	44
J.F.	ASD	33	M	69	136	1.74	3,540	109	1,710	157	5,250	121	1,510	139	6,760	124	22
K.B.	VSD§	21	F	63	147	1.68	2,300	91	1,080	129	3,340	100	840	100	4,180	100	20
M.C.	VSD	32	F	64	104	1.47	1,780	81	1,180	162	2,960	101	770	105	3,730	102	21
J.C.	VSD	19	M	70	152	1.85	3,450	100	1,530	132	4,980	108	870	75	5,850	101	15
P.D.	VSD	19	F	62	130	1.58	2,290	97	1,240	157	3,530	112	1,530	194	5,060	128	30
W.D.	VSD	33	M	68	142	1.74	2,420	74	1,540	141	3,960	91	2,080	191	6,040	111	34
J.F.	VSD	19	M	70	141	1.78	2,540	76	1,490	134	4,030	91	1,330	120	5,360	96	25
J.G.	VSD	32	M	71	167	1.94	3,320	91	2,200	182	5,480	113	1,960	162	7,440	123	26
B.H.	VSD	40	F	64	120	1.57	2,910	124	840	106	3,500	111	1,650	171	5,150	125	32
D.H.	VSD	18	F	65	136	1.67	2.080	83	1,400	166	3,480	104	1,150	151	4,630	111	25
C.J.	VSD	24	M	72	161	1.94	2,970	82	1,640	136	4,610	95	3,160	261	7,770	128	41
G.P.	VSD	32	F	64	117	1.56	2,120	91	1,310	168	3,170	102	1,290	165	4,460	114	29
Mean		25				1.68		89		148		103		170		116	29

*Per cent of predicted value based on age and surface area.
†Residual volume/total lung capacity ratio × 100.
‡Atrial septal defect.
§Ventricular septal defect.

per minute for women and 250 ml. per minute for men. Arterial blood was drawn from the brachial artery through an indwelling needle. Mixed venous blood for the pulmonary blood flow was obtained from the pulmonary artery through an indwelling cardiac catheter at the same time the arterial sample was taken and oxygen consumption was being measured. In patients with atrial septal defect, the mixed venous blood value used in calculating the systemic blood flow was the average of blood samples obtained through the cardiac catheter from the superior vena cava and inferior vena cava 10 to 30 minutes before cardiac output determination. In patients with ventricular septal defect, the average of blood samples obtained from the right atrium was used as the mixed venous blood value. Per cent of shunt was calculated using the following formula:

$$\text{Per cent shunt} = \frac{\text{pulmonary blood flow} - \text{systemic blood flow}}{\text{pulmonary blood flow}}$$

TABLE III. STUDIES OF ALVEOLAR GAS DISTRIBUTION, RESPIRATORY MECHANICS, AND DIFFUSION IN NORMAL SUBJECTS

	ALVEOLAR GAS DISTRIBUTION		MECHANICAL TESTS				DIFFUSION	
			MAXIMAL BREATHING CAPACITY		MAXIMAL EXPIRATORY FLOW RATE	MAXIMAL INSPIRATORY FLOW RATE		
PATIENT	7 MIN. WASHOUT (% N_2)*	SINGLE BREATH N_2 TEST (% N_2)†	L./MIN.	%‡	(L./MIN.)	(L./MIN.)	ML. CO/MM. Hg/MIN.	%§
R.C.	0.5	0.4	131	88	363	255	27	91
W.C.	0.4	0.1	129	93	400	293	36	127
J.K.	0.2	0.2	158	111	364	400	32	112
J.M.	0.3	1.4	131	102	286	311	33	131
R.R.	0.5	0.6	179	138	480	387	31	109
L.S.	0.2	0.8	189	129	333	261	37	124
R.S.	0.5	0.9	155	108	480	316	29	97
B.S.	0.4	0.5	167	127	387	266	24	90
J.S.	0.5	0.3	156	99	631	293	30	89
H.W.	0.5	0.3	142	111	308	250	26	90
Mean	0.4	0.6		110	403	303	31	106

*Normal values for 7 minute nitrogen washout are less than 2.5 per cent N_2.
†Normal values for single breath nitrogen test are less than 1.5 per cent N_2.
‡Per cent of predicted normal based on age and surface area.
§Per cent of predicted normal based on surface area.

RESULTS

Ten normal persons and twenty patients with intracardiac shunts were studied. The normal subjects were students with a mean age of 22 years and a mean body surface area of 1.90 square meters. The patients with septal defects were four men and five women with atrial septal defect and five men and six women with ventricular septal defect. The mean age was 25 years, the mean body surface area 1.68 square meters.

A. *Lung Volumes.*—The values for lung volumes are shown in Tables I and II. The mean vital capacity was 104 per cent of predicted normal in the normal subjects and 103 per cent of predicted normal in patients with intracardiac shunts. The mean residual volume was 129 per cent of predicted normal in the normal group and 170 per cent of predicted normal in the patients with intracardiac shunts. This is not a significant difference ($p > 0.1$). The

TABLE IV. STUDIES OF VENTILATION, RESPIRATORY MECHANICS, AND DIFFUSION IN PATIENTS WITH INTRACARDIAC SHUNTS

		VENTILATION						MECHANICAL TESTS				DIFFUSION	
		MINUTE VOLUME		PHYSIOLOGIC DEAD SPACE		ALVEOLAR GAS DISTRIBUTION		MAXIMAL BREATHING CAPACITY					
PATIENT	CLINICAL DIAGNOSIS	TOTAL (L.)	ALVEOLAR (L.)	ML.	%*	7 MIN. WASHOUT (% N_2)†	SINGLE BREATH N_2 TEST (% N_2) ‡	L./MIN.	%§	MAXIMAL EXPIRATORY FLOW RATE (L./MIN.)	MAXIMAL INSPIRATORY FLOW RATE (L./MIN.)	ML. CO/MM. Hg/MIN.	%‖
M.C.	ASD	13.7	8.7	278	37	0.3	0.7	131	96	214	103	51	185
M.Mc.	ASD	7.3	4.0	143	45	0.3	1.5	98	103	267	182	23	100
B.P.	ASD	9.0				0.4	1.4	100	124	207	156	45	199
D.P.	ASD	8.2	5.7	301	31	0.3	0.8	181	111	387	293	46	140
E.R.	ASD	11.0	7.6	238	31	1.1	1.4	119	108	353	255	52	206
S.D.	ASD	7.2	4.8	103	33	0.2	0.5	94	97	222	134	34	158
B.M.	ASD	8.4	5.4	135	36	0.4	1.2	88	93	250	222	26	122
G.V.	ASD	8.2				0.4	1.3	106	120	194	171	40	200
J.F.	ASD	9.9	7.5	143	24	1.0	1.4	125	103	194	160	45	173
K.B.	VSD	8.1				0.5	1.0	109	106	211	160	32	129
M.C.	VSD	7.4	4.3	143	42	0.8	1.5	70	78	164	115	18	86
J.C.	VSD	9.5	5.9	169	38	0.3	0.8	180	127	324	267	37	132
P.D.	VSD	4.8	2.4	120	50	0.3	0.4	132	135	226	176	30	130
W.D.	VSD	9.4	5.5	202	42	6.0	4.6	105	87	279	250	28	108
J.F.	VSD	8.6	5.2	176	40	0.5	0.3	110	81	333	164	31	116
J.G.	VSD	8.6	4.5	241	48	0.6	1.0	137	101	343	179	38	128
B.H.	VSD	13.0	7.0	203	46	1.0	1.1	74	83	207	140	26	114
D.H.	VSD	4.6	3.1	234	49	0.8	1.4	96	91	230	136	30	121
C.J.	VSD	8.1	4.7	248	42	0.3	0.5	120	83	240	211	32	107
G.P.	VSD					0.6	0.5	96	110	261	211	34	150
Mean		8.7	5.4	192	40	0.8	1.2		102	255	181	35	140

*Per cent of tidal volume.
†Normal values for 7 minute nitrogen washout are less than 2.5 per cent N_2.
‡Normal values for single breath nitrogen test are less than 1.5 per cent N_2.
§Per cent of predicted normal based on age and surface area.
‖Per cent of predicted normal based on surface area.

mean total lung capacity was 110 per cent of predicted normal in normal subjects and 116 per cent of predicted normal in patients with intracardiac shunts. This is not a significant difference ($p > 0.2$).

B. *Ventilation.*—The values for the minute volume of total ventilation, the minute volume of alveolar ventilation, and the physiologic dead space in patients with intracardiac shunts were normal (Table IV). Distribution of inspired air as measured by the single breath nitrogen test was not significantly different in normal subjects (Table III) from that in patients with intracardiac shunts (Table IV) ($p > 0.05$).

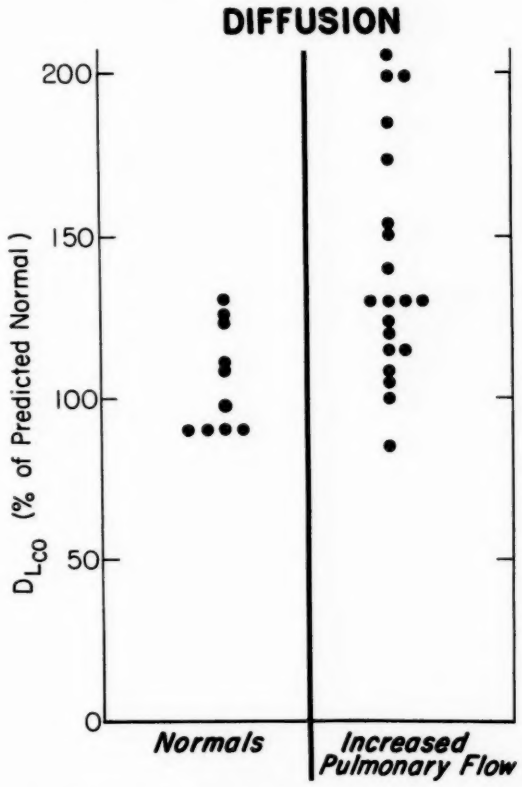

Fig. 1.—The D_{LCO} in resting normal subjects and in patients with intracardiac shunts plotted as per cent of predicted normal.

C. *Mechanical Tests.*—The values for maximal breathing capacity and maximal flow rates are shown in Tables III and IV. The mean maximal breathing capacity was 110 per cent of predicted normal in the normal group and 102 per cent of predicted normal in the patients with intracardiac shunts. This is not a significant difference ($p > 0.6$). The mean maximal expiratory flow rate was 403 L. per minute in the normal subjects and 255 L. per minute in patients with intracardiac shunts. This is a significant difference ($p < 0.01$). The mean maximal inspiratory flow rate was 303 L. per minute in normal subjects and 181 L. per minute in patients with intracardiac shunts. This is not a significant difference ($p > 0.05$).

TABLE V. ARTERIAL BLOOD STUDIES AND CARDIAC CATHETERIZATION FINDINGS IN PATIENTS WITH ATRIAL SEPTAL DEFECT AND VENTRICULAR SEPTAL DEFECT

PATIENT	CLINICAL DIAGNOSIS	O_2 CONTENT (REST) (VOL. %)	O_2 CAPACITY (VOL. %)	O_2 SATURATION		P_{CO_2} (REST) (MM. Hg)	pH (REST)	HEMATOCRIT (%)	RIGHT VENTRICULAR PRESSURE (MM. Hg)	PULMONARY ARTERIAL PRESSURE (MM. Hg)	ESTIMATED PULMONARY FLOW (L./MIN.)	ESTIMATED SYSTEMIC FLOW (L./MIN.)	SHUNT (%)
				REST (%)	100% O_2* (%)								
M.C.	ASD	19.53	19.96	97.9	100 + 1.48	38	7.40	46		25/8	16.2	4.6	72
M.Mc.	ASD	17.75	17.79	99.8	100 + 1.92	32	7.47	45	42/10	40/18	6.5	3.0	50
B.P.	ASD	16.80	17.10	99.3				42	30/0	27/10	18.2	3.3	80
D.P.	ASD	19.85	19.89	99.8	100 + 3.35	43	7.33	49	40/0	27/12			
E.R.	ASD	16.67	16.67	100.0	100 + 2.20	33	7.48	43	31/0	33/10	28.7	4.5	84
S.D.	ASD	18.01	18.68	96.2	100 + 2.26	37	7.46	44	30/0	30/11	7.5	3.5	55
B.M.	ASD	15.64	15.42	100 + 0.22	100 + 2.31	31	7.44	37	23/0	20/7	8.8	4.3	51
G.V.	ASD	18.31	18.49	99.0				44	30/0	30/10	9.5	4.3	55
J.F.	ASD	19.37	18.90	100 + 0.47	100 + 2.76	32	7.45	48	40/0	36/10	16.8	3.9	77
K.B.	VSD	15.05	15.22	98.9	100 + 1.83			36	35/0	27/9	11.1	5.8	48
M.C.	VSD	14.30	14.89	96.0	100 + 1.46	36	7.39	39		33/14	13.3	4.9	63
J.C.	VSD	18.34	18.61	98.6	100 + 2.61	39	7.43	46	34/2	30/13	8.1	3.8	53
P.D.	VSD	16.80	17.27	97.3	100 + 1.90	34	7.41	41		23/9	8.6	6.7	22
W.D.	VSD	18.48	18.69	98.9	100 + 1.51	30	7.50	47	30/0	32/10	10.1	5.1	50
J.F.	VSD	17.73	17.77	99.8	100 + 2.02	39	7.44	44	54/0	40/10	19.7	3.8	81
J.G.	VSD	19.42	20.53	94.6	100 + 0.85	43	7.35	47	24/2	21/14	6.9	3.4	51
B.H.	VSD	16.53	15.55	100 + 0.98	100 + 2.42	32	7.42	40	24/1	19/7	5.3	4.0	25
D.H.	VSD	16.94	17.70	100 + 0.30	100 + 2.25	40	7.39	44	27/2	23/8	6.2	4.6	26
C.J.	VSD	18.79	18.26	100 + 0.53	100 + 2.35	49	7.33	48		25/9	6.8	5.7	16
G.P.	VSD	16.38	16.12	100 + 0.26				40	22/5	20/11	6.4	3.8	41
Mean				98.9	100 + 2.09†	37‡	7.42‡	44			11.3§	4.4§	53§

*Values following the + sign refer to milliliters O_2 per 100 ml. blood in excess of that required to saturate hemoglobin (i.e., dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.

†Mean value represents only seventeen patients.

‡Mean value represents only sixteen patients.

§Mean value represents only nineteen patients.

D. *Diffusion.*—The values for $D_{L_{CO}}$ are plotted in Fig. 1 and shown in Tables III and IV. The mean value in normal subjects was 31 ml. carbon monoxide (CO) per millimeter mercury per minute or 106 per cent of predicted normal. The mean value in patients with intracardiac shunts was 35 ml. CO per millimeter mercury per minute or 140 per cent of predicted normal. The groups are significantly different when the values expressed in terms of per cent of predicted normal are compared ($p < 0.01$). The mean value for diffusion in patients with atrial septal defect was 40 ml. CO per millimeter mercury per minute or 165 per cent of predicted normal; the mean value in the patients with ventricular septal defect was 31 ml. per millimeter mercury per minute or 120 per cent of predicted normal. This difference is statistically significant ($p < 0.01$).

E. *Arterial Blood Studies.*—The results of arterial blood studies in patients with intracardiac shunts are shown in Table V. Arterial oxygen saturation, arterial P_{CO_2}, and arterial pH were normal. No patient had evidence of right-to-left shunt.

F. *Cardiac Catheterization Findings.*—Right ventricular pressure, pulmonary arterial pressure, estimated pulmonary blood flow, estimated systemic blood flow, and per cent of shunt are listed in Table V.

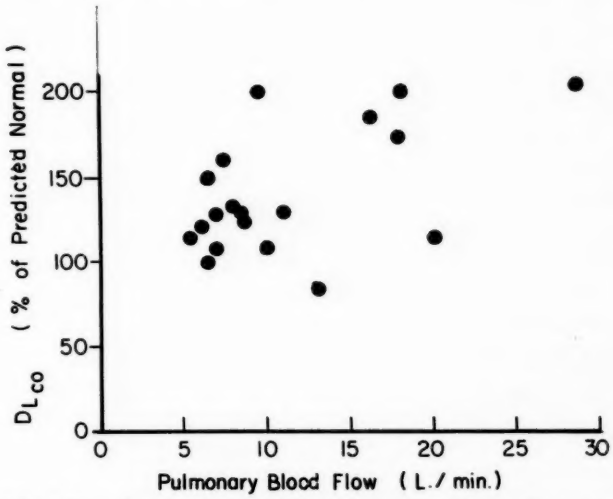

Fig. 2.—A plot of $D_{L_{CO}}$ (per cent of predicted normal) against pulmonary blood flow in patients with intracardiac shunts.

DISCUSSION

These studies demonstrate a significantly higher $D_{L_{CO}}$ in a group of patients with intracardiac shunts than in a comparable group of normal subjects. A plot of $D_{L_{CO}}$ against pulmonary blood flow (Fig. 2) shows that there is a tendency for $D_{L_{CO}}$ to be greater as flow increases. Auchincloss and associates[14] and Rankin and Callies[15] have found increased $D_{L_{CO}}$ in patients with intracardiac shunts. What is the explanation for these findings? The $D_{L_{CO}}$ is dependent on the total capillary surface available for and actively involved in

diffusion, the thickness and character of the alveolar capillary membrane, and the reaction rate of carbon monoxide with hemoglobin.[16, 17] The reaction rate of carbon monoxide with hemoglobin is slowed in the presence of anemia,[18] and probably accelerated by polycythemia. The hematocrit in the group with intracardiac shunts was normal. Hematocrits were not measured in the normal subjects but are presumed to be normal. The difference in $D_{L_{CO}}$ in the two groups cannot be satisfactorily explained by alteration in the reaction rate of carbon monoxide with hemoglobin. $D_{L_{CO}}$ is altered if the character or thickness of the alveolar capillary membrane is changed. Because the two groups were comparable in age and because the patients had normal or very slightly elevated pulmonary arterial pressure, it is unlikely that there was a significant difference in the character or thickness of the alveolar capillary membrane. This leaves the extent of alveolar capillary membrane available for and actively participating in diffusion. An increase in capillary surface participation in diffusion seems the best explanation for the increased $D_{L_{CO}}$ in the patients with intracardiac shunts. The capillary surface area must in turn be related to the pulmonary capillary blood volume. I do not have measurements of pulmonary capillary blood volume but Rankin and Callies[15] do. They have found it to be increased in patients with intracardiac shunts.

The effect of increased pulmonary blood flow on pulmonary diffusing capacity is not completely understood. That diffusion increases with exercise is well known. This has been thought to be related to increased cardiac output. In some studies, however, increased pulmonary blood flow has not been associated with any change in diffusion. Ross and collaborators[19] increased cardiac output in normal resting subjects by three methods: drugs, albumin infusion, and release of arterial tourniquets which had occluded blood flow into the lower extremities. They found no increase in diffusing capacity when cardiac output was increased by these methods. Johnson and co-workers[20] found that the diffusing capacity of several patients with increased cardiac output caused by hyperthyroidism was not significantly different from their diffusing capacity when cardiac output had returned to normal after treatment. Turino and associates[21] increased blood flow through one lung by occluding the pulmonary artery on the other side in patients with various types of lung disease. They found no increase in diffusing capacity on the side of increased blood flow.

On theoretic grounds, pulmonary blood flow should have little relationship to $D_{L_{CO}}$ as long as the capillary bed remains normal in size. In measuring $D_{L_{CO}}$ the speed of blood flow past the pulmonary alveolar capillary membrane is not critical. The hemoglobin "compartments" for carbon monoxide are so large that they cannot possibly be filled by the molecules of carbon monoxide that diffuse across the alveolar capillary membrane in the 10 seconds necessary for the test. Even if pulmonary blood flow stopped, transfer of carbon monoxide from the alveolus to hemoglobin would continue for a minute or more before the pockets became full.[22] Therefore, if the increased cardiac output flows rapidly through a capillary bed of normal size, the $D_{L_{CO}}$ will be normal.

This would explain the findings in the patients with thyrotoxicosis studied by Johnson and collaborators,[20] the normal $D_{L_{CO}}$ in the face of increased cardiac output found by Ross and co-workers,[19] and the failure of $D_{L_{CO}}$ to increase in one lung after occlusion of the pulmonary artery on the opposite side found by Turino and associates.[21] If increased pulmonary blood flow is associated with enlargement of the capillary bed, the $D_{L_{CO}}$ will be high. This would explain the results on exercise[16] and in patients with intracardiac shunts. The mechanism which controls capillary bed size is unknown. This must be independent of cardiac output in order to explain the diverse findings in regard to $D_{L_{CO}}$ when cardiac output is increased. Elevation of pressure somewhere between the pulmonary valve and the aortic valve might be the mechanism of control. I suspect that the main pulmonary artery is not the site of this regulatory activity because in the patients with intracardiac shunts main pulmonary arterial pressure was normal.

SUMMARY AND CONCLUSIONS

The diffusing capacity for carbon monoxide ($D_{L_{CO}}$) is significantly higher in resting patients with intracardiac septal defects than in resting normal subjects. The diffusing capacity for carbon monoxide in patients with atrial septal defect is significantly higher than in those with ventricular septal defect. The increased $D_{L_{CO}}$ is best explained by an increase in pulmonary surface area available for diffusion.

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ERYTHROPOIETIN PRODUCTION IN UREMIC RABBITS

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Certain experimental observations have indicated that the kidney might elaborate a humoral substance, erythropoietin, which participates in the regulation of red cell production. Experiments reported in this article were designed to assess and compare erythropoietin production in intact and nephrectomized rabbits and in rabbits with uremia induced by ureteral ligation. Hypoxia for 8 hours in a low atmosphere chamber or anemia induced by bleeding was used to stimulate erythropoietin production.

Plasmas recovered from the various rabbits were extracted and bioassayed for erythropoietin content. Assays were carried out in female rats that had been rendered sensitive to exogenous erythropoietin by transfusion induced polycythemia. The 18 hour red cell radioiron incorporation in the assay rat was the parameter used to measure erythropoietin content of the various extracts.

Polycythemia reduced Fe^{59} incorporation from the normal 27.8 ± 1.02 per cent to 6.2 ± 0.2 per cent in the control assay rat. Injection of hypoxic or anemic plasma extracts increased iron incorporation from 6.2 per cent to 19.6 ± 2.29 per cent and 25.0 ± 1.84 per cent, respectively. Polycythemic rats injected with hypoxic nephrectomized rabbit plasma extract consistently incorporated more Fe^{59} (9.5 ± 0.44 per cent) than did rats injected with normal plasma extract (5.7 ± 0.57 per cent) or extract of plasma from nonstimulated nephrectomized rabbits (5.0 ± 0.3 per cent). Anemia produced less significant increases in the plasma erythropoietin content of nephrectomized rabbits (8.3 ± 1.16 per cent).

These results indicate that the nephrectomized rabbit can significantly increase plasma levels of erythropoietin in response to hypoxia and anemia. Thus the kidney cannot be the only source of this hormone in the rabbit.

REPEATED observations have verified the hypothesis that red cell production is regulated in part by a humoral substance. Experimentally induced anemia or hypoxia results in the appearance of this substance(s) in the plasma of the stimulated animal and appropriate bioanalytical methods can then be employed to demonstrate indirectly the erythropoiesis enhancing effect of this stimulated or active plasma. Similarly, plasma from certain anemic or hypoxic[1-3] humans has an erythropoiesis stimulating effect. The origin of the active factor(s), "erythropoietin(s)," remains in doubt, but the observations of Jacobson and co-workers[4-7] strongly support the thesis that the kidney either produces or activates erythropoietin. In addition, Reichlin and collaborators[8] assayed levels of erythropoietin in plasma from nephrectomized rats and found

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these to be lower than the levels found in normal rats. Other observations,[9-12] however, are at variance with the concept of a singular renal origin for erythropoietin.

Experiments to be reported in this article were designed to assess and compare erythropoietin production in intact and in nephrectomized rabbits. Extracts of plasma from normal rabbits, hypoxic intact rabbits, hypoxic nephrectomized rabbits, and nonstimulated nephrectomized rabbits were bioassayed for erythropoietin content. The effect of uremia induced by ureteral ligation on plasma erythropoietin levels was also investigated. Finally an effort was made to determine if the activity of erythropoietin could be altered by substances present in uremic plasma.

METHODS AND MATERIALS

New Zealand albino rabbits weighing 3 to 4 kilograms were used as plasma donors. All plasma samples were collected by cardiac puncture with pentobarbital anesthesia. Heparin was used as an anticoagulant and the various plasmas were stored frozen at −18° C. until the time of extraction. This latter procedure was a modification of the method of Borsook and associates[13] and the one previously reported from this laboratory.[3] In most experiments the supernatants collected after plasma extraction were concentrated by flash evaporation to one third the original plasma volume. As will be shown, assay of the threefold concentrated extracts did not demonstrate any difference in activity between normal plasma and plasma from nephrectomized rabbits. Therefore, in a single series of experiments more highly concentrated extracts were produced by evaporation to one tenth of the original plasma volume. These more concentrated extracts were dialyzed against distilled water at 4° C. for 12 hours prior to assay. All extracts were stored frozen until time of assay.

Two methods of bioassay were employed. Polycythemic rats have been used previously[14] in the bioassay of erythropoietin and were utilized in the majority of the present experiments. These assay animals were prepared by transfusing 160 to 180 gram female Sprague-Dawley rats* with a single injection of an 80 per cent suspension of homologous rat red cells in the amount of 1.4 ml. per 100 grams of weight. Twenty-four and 48 hours after transfusion, 2 ml. of the test plasma extract was given intravenously to each rat. On the following day approximately 1 μc Fe^{59} citrate in saline was given by the same route. The 18 hour red blood cell Fe^{59} incorporation was then determined and used as an indirect measure of erythropoietin level of the extract. The hematocrits of all polycythemic assay rats were determined at the end of each experiment to exclude falsely high values of iron incorporation caused by bleeding or hemolysis. Each extract was bioassayed in a group of five rats; the mean red cell iron incorporation for the group denoted the activity of the extract under study. Starved rats were used as assay animals in certain experiments as will be specified. This assay method, described by Fried,[14] has been used extensively in our laboratory during the past 2 years.

Experiments, designed to ascertain the effect of nephrectomy or ureteral ligation on erythropoietin production in hypoxic rabbits, were carried out by assaying and comparing the erythropoietic stimulating activity of the plasma of rabbits that had been subjected to the various procedures. Control experiments were conducted by assaying plasma from either normal rabbits or rabbits that had been nephrectomized 24 hours prior to sacrifice. Similar groups of normal rabbits and nephrectomized rabbits were subjected to 8 hours of hypoxic stimulation prior to sacrifice. In addition, rabbits rendered uremic by ureteral ligation were similarly subjected to 8 hours of hypoxia. Plasmas from these different animal preparations were harvested, extracted, and assayed in polycythemic rats.

*Holtzman strain, Madison, Wis.

Hypoxia was induced in each instance by subjecting the various animals to a simulated altitude of 22,000 feet for 8 hours in a low pressure chamber. The animals which had been operated upon were allowed 16 hours for recovery before induction of hypoxia. In most experiments intact and nephrectomized animals were subjected to hypoxia concomitantly. All uremic animals were autopsied after exsanguination and the hematocrit and blood urea nitrogen of each animal were determined by standard techniques.

In order to assess the effect of a different erythropoietic stimulus, seven nephrectomized rabbits were bled 20 ml. per kilogram immediately after operation. Saline in comparable volume was infused to prevent shock and the animals were killed 24 hours after nephrectomy. Plasma was collected, prepared by extraction as described, and stored frozen until assayed in polycythemic rats.

In certain experiments, erythropoietin-rich plasma was recovered from rabbits with a hemolytic anemia induced by serial daily injections of 1 ml. of a 2.5 per cent solution of phenylhydrazine. Equal amounts of this active plasma were mixed with varying amounts of uremic plasma harvested from nephrectomized rabbits. Combined plasmas were incubated for 2 hours at 37° C., extracted, and assayed in starved rats. An unaltered sample of equal volume of the same anemic plasma was assayed as a control. The urea nitrogen content of the anemic plasma, the uremic plasma, and the mixed plasmas was determined.

In another experiment, erythropoietin-rich plasma from phenylhydrazine treated rabbits was divided into two pools. One pool was altered by the addition of uremic plasma and incubated at 37° C. for 8 hours. Samples of this mixture were removed at 0, 2, 4, 6, and 8 hours. Extraction was carried out immediately. The unaltered pool of the same erythropoietin-rich plasma was also incubated and samples were removed at similar intervals to serve as controls. All these extracts were prepared in the usual fashion and assayed in starved rats.

RESULTS

Transfusion induced polycythemia or starvation for 96 hours partially arrested erythropoiesis in the assay rats and reduced the eighteen hour red cell radioiron incorporation to a level below that found in normal rats. Table I shows the comparative erythrocyte Fe^{59} incorporation in normal, polycythemic, and starved rats injected with saline. The polycythemic or starved animals have diminished erythropoiesis but are capable of increasing red cell radioiron incorporation in response to the administration of exogenous erythropoietin; they were used in bioassays designed to compare the erythropoietin content of various plasmas. The results of assays in polycythemic rats of extracts obtained from the plasma of normal, hypoxic, or anemic rabbits are recorded in Part A of Table II. The red cell radioiron incorporation in polycythemic rats injected with extracts of normal plasma was 5.7 ± 0.57 per cent, whereas iron incorporation in rats injected with hypoxic or anemic rabbit plasma extracts was 19.6 ± 2.29 per cent and 25.0 ± 1.84 per cent, respectively. A similar responsiveness to exogenous erythropoietin has been found in starved rats injected with active plasma extracts. Extensive experiments undertaken in this laboratory[15] to test

TABLE I. EIGHTEEN HOUR RED CELL RADIOIRON INCORPORATION IN NORMAL AND ASSAY RATS INJECTED WITH SALINE

ASSAY ANIMAL	MATERIAL INJECTED	EXPERIMENTS	NO. OF ANIMALS	Fe^{59} INCORPORATION*
Normal	Saline	11	56	27.84 ± 1.02
Starved	Saline	32	166	4.83 ± 0.16
Polycythemic	Saline	21	111	6.2 ± 0.20

*Per cent Fe^{59} incorporation in 18 hours in erythrocytes of assay or normal rats ± standard deviation of the mean.

the comparative sensitivity of these two types of assay animals have shown no essential difference in response when the same dose of erythropoietin was injected.

Induction of uremia, by ureteral ligation or nephrectomy prior to hypoxic stimulation, apparently reduced the animals' response to hypoxia and led to lower levels of plasma erythropoietin in the uremic animals after stimulation than were found in the hypoxic intact rabbits (Table II, Part B). Assay rats injected with extracts of plasma from rabbits with ureters ligated prior to hypoxia had an Fe^{59} uptake of 13.9 ± 1.57 per cent whereas similar rats injected with extracts of plasma from hypoxic nephrectomized rabbits incorporated 9.5 ± 0.44 per cent of the radioiron in their erythrocytes. This is in contrast to an Fe^{59} incorporation of 19.6 per cent in rats injected with plasma extracts from hypoxic intact rabbits.

TABLE II. EIGHTEEN HOUR RED CELL RADIOIRON INCORPORATION IN POLYCYTHEMIC RATS INJECTED WITH THREEFOLD CONCENTRATED RABBIT PLASMA EXTRACT

MATERIAL INJECTED	NO. OF RABBITS*	Fe^{59} INCORPORATION†
Part A.—		
Normal plasma extract	8	5.7 ± 0.57
Hypoxic plasma extract	8	19.6 ± 2.29
Anemic plasma extract	5	25.0 ± 1.84
Part B.—		
Nephrectomized nonstimulated plasma extract	9	5.0 ± 0.3
Nephrectomized hypoxic plasma extract	9	9.5 ± 0.44
Ureter ligated hypoxic plasma extract	6	13.9 ± 1.57
Nephrectomized anemic plasma extract	7	8.3 ± 1.16

*Plasma from each rabbit assayed in 5 rats.

†Per cent Fe^{59} incorporation in 18 hours in erythrocytes of assay rats ± standard deviation of the mean.

Erythropoietin production in response to hypoxia was reduced when nephrectomy or ureteral ligation was carried out prior to stimulation. Nevertheless, the levels of plasma erythropoietin in the nephrectomized animals after hypoxia were higher than the levels found in nonstimulated nephrectomized rabbits. The mean iron incorporation of 9.5 ± 0.44 per cent in the rats injected with extracts of plasma from hypoxic nephrectomized rabbits was significantly higher than the iron incorporation of 5.0 ± 0.3 per cent found in assay rats injected with extracts of plasma from nephrectomized nonstimulated rabbits ($p < 0.001$). The latter figure is not significantly different from the 5.7 ± 0.57 per cent obtained after injection of normal plasma extracts.

When assay rats were injected with extracts of plasma from anemic nephrectomized rabbits they incorporated 8.3 ± 1.16 per cent of the Fe^{59} in their erythrocytes in 18 hours (Table II). When this was compared to the 5.0 ± 0.3 per cent iron incorporation in rats injected with extract of nephrectomized nonstimulated rabbit plasma, the difference was less significant ($p < 0.008$) than the difference obtained when hypoxia was used for stimulation. The mean blood urea nitrogen in hypoxic-nephrectomized rabbits 24 hours after removal

TABLE III. EIGHTEEN HOUR RED CELL RADIOIRON INCORPORATION IN POLYCYTHEMIC RATS INJECTED WITH TENFOLD CONCENTRATED RABBIT PLASMA EXTRACT

MATERIAL INJECTED	NO. OF EXPERIMENTS	Fe^{59} INCORPORATION*
Normal plasma extract	4†	5.4 ± 0.49
Nephrectomized nonstimulated plasma extract	5	5.9 ± 0.64
Nephrectomized hypoxic plasma extract	5	13.7 ± 1.11

*Per cent Fe^{59} incorporation in 18 hours in erythrocytes of assay or normal rats ± standard deviation of the mean.

†In each experiment plasma was obtained from three or four rabbits, pooled to 200 ml., extracted reconstituted to 1/10 the original volume and assayed in a group of 5 rats.

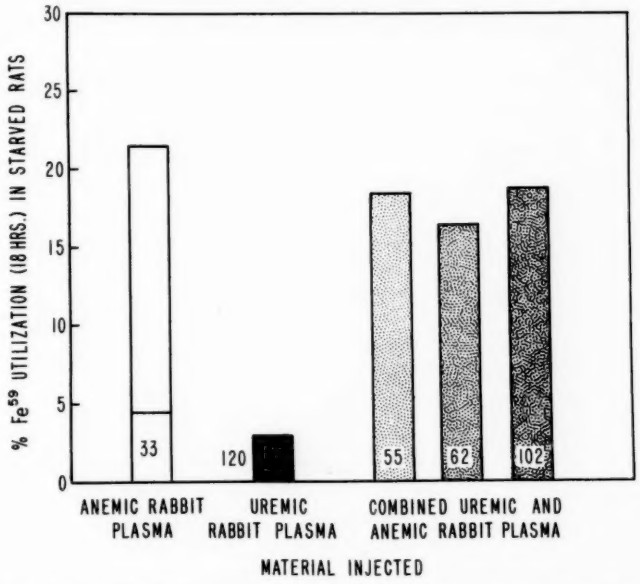

Fig. 1.—Erythropoietin content of active anemic plasma before and after combination and 2 hour incubation with uremic plasma. The numbers in each bar indicate the urea nitrogen level of the original and combined plasmas.

of the kidneys was 94.1 mg. per cent whereas the blood urea nitrogen 24 hours after ureteral ligation was 87.6 mg. per cent. Normal rabbits have a blood urea nitrogen of approximately 15 mg. per cent.

Table III records the results of the assays on tenfold concentrated rabbit plasma extract. The 18 hour red cell Fe^{59} incorporation was 13.8 ± 1.11 per cent in polycythemic assay rats injected with the more highly concentrated extracts of plasma from hypoxic nephrectomized rabbits. On assay these more highly concentrated extracts demonstrated an increased difference in the erythropoietic stimulating activity of plasma from nephrectomized rabbits with and without hypoxic stimulation. No activity was demonstrable in normal plasma, so concentrated, and there was no significant difference between the Fe^{59} incorporation of the rats injected with these plasma extracts and rats injected with extracts of plasma from nephrectomized nonhypoxic rabbits.

Fig. 1 shows the results of assay of anemic rabbit plasma before and after it was rendered uremic by the addition of plasma from nephrectomized rabbits. In this experiment a pool of plasma from rabbits with a phenylhydrazine-induced

hemolytic anemia was divided into four equal parts. One fraction was extracted without further alteration. The remaining three fractions were combined with increasing amounts of uremic plasma with a urea nitrogen content of 120 mg. per cent, incubated in vitro for 2 hours at 37° C., and extracted. All extracts were assayed in starved rats. As is seen in Fig. 1, the unaltered anemic plasma extract increased Fe^{59} incorporation in the starved rat from 4 to 21.4 per cent whereas combined plasma extracts with urea nitrogen concentrations of 55, 62, and 102 mg. per cent stimulated red cell radioiron incorporation to 18.6, 16.5, and 18.9 per cent, respectively. Although there was slight loss of activity in the combined plasma, it could not be directly correlated with the amount of added uremic plasma.

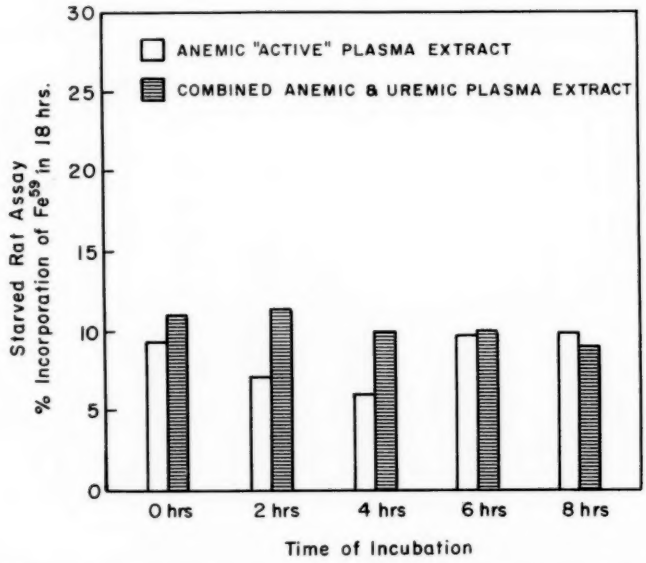

Fig. 2.—Erythropoietin content of unaltered anemic plasma and combined anemic and uremic plasma incubated for 8 hours.

In a final experiment active anemic plasma, with urea nitrogen content of 30 mg. per cent, was divided into two equal pools. One pool was left unaltered whereas that remaining was combined with uremic plasma resulting in a mixed plasma with urea nitrogen content of 74 mg. per cent. Both the anemic plasma and the combined plasma were incubated in vitro at 37° C. for 8 hours. The pH of both plasmas did not vary significantly from 7.8 during the 8 hour incubation. As is shown in Fig. 2, serial removal and assay of samples containing equal amounts of the active plasma from each plasma pool showed no significant alteration of erythropoiesis stimulating activity that could be related to the contents of the uremic plasma. There was some variation in the activity of the 2 and 4 hour control plasma extracts.

DISCUSSION

The role of the kidney in the regulation of erythropoiesis continues to stimulate great interest. Although certain clinical observations[16-22] support the

concept that an erythropoiesis stimulating factor (erythropoietin) may be elaborated by the kidney, the real foundation for this concept is provided by the experimental observations of Jacobson and associates[1-7] and a number of other investigators.

Originally Jacobson and coauthors[1] reported that normal rats injected with cobalt or made anemic by bleeding responded by increasing their plasma levels of erythropoietin. This response was completely blocked if nephrectomy was effected prior to the stimulation by bleeding or cobalt injection but only partially blocked with the induction of a similar degree of uremia by means of bilateral ureteral ligation. Mirand and Prentice[9] were able to demonstrate increased plasma levels of erythropoietin in nephrectomized rats subjected to hypoxia, but Jacobson and co-workers could not confirm[6] the latter observations when they measured red cell Fe^{59} incorporation in starved rats injected with plasma from hypoxic nephrectomized rats. However, assay of the hypoxic rat plasma in polycythemic mice[7] did produce a slight increase in the reticulocyte level of these assay animals.

Further experimental evidence for a renal origin of erythropoietin is supported by the observation of Reichlin and associates,[8] van Lessen and coworkers,[22] and Naets.[23, 24] Reichlin[8] found lower levels of plasma erythropoietin in nephrectomized rats than were found in normal rats. Van Lessen produced a temporary erythrocytosis in dogs by partial unilateral renal vein ligation and concluded that the engorged kidney might produce an erythropoietic factor. Naets[23] assayed the erythropoietic effect of homogenates of anemic dog kidney and demonstrated a slightly higher activity in this preparation than he found in homogenates of normal kidney or anemic liver or spleen. In other experiments, Naets[24] found that a nephrectomized dog was able to maintain a normal erythropoietic marrow when injected with erythropoietin in spite of severe uremia. He concluded that the cessation of erythropoiesis after nephrectomy was due to the absence of the kidney factor and not to uremia itself.

Osnes,[11] on the other hand, concluded from his observations that erythropoietin was produced in an extrarenal site but that the kidney did indeed produce a different erythropoietic principle. In his opinion, a deficiency of this second principle accounts for the anemia seen in patients with chronic nephritis.

Erslev[10] studied erythropoietin production in rabbits. Initially he produced a standard reticulocyte and bone marrow normoblast response in normal animals by subjecting them to bleeding. This erythropoietic response was completely abolished if the rabbits were made uremic by either nephrectomy or ureteral ligation prior to the stimulation by bleeding. Nonetheless, plasma harvested from nephrectomized rabbits 20 hours after the same standard bleeding proved to have an erythropoietin content equal to that of plasma from normal bled animals when both were assayed in intact rabbits. Thus Erslev concluded that the block in erythropoiesis was due to uremic suppression and not to the absence of kidney tissue or impaired erythropoietin production.

The results of the studies presented in this report tend to parallel closely those of Erslev. Rabbits rendered uremic by either nephrectomy or ureteral

ligation did have lower plasma erythropoietin levels after hypoxia than did the intact animal (Table II). However, plasma from hypoxic nephrectomized rabbits had a significantly higher erythropoietin content than did the plasma of normal rabbits or nephrectomized rabbits which had not been stimulated by hypoxia (Tables II and III). Bleeding proved to be a less potent stimulus for erythropoietin production (Table II) than did hypoxia.

Attempts to demonstrate lower levels of erythropoietin in nephrectomized rabbit plasma than in normal plasma proved unsuccessful in spite of tenfold concentration of the plasma extract. This increased concentration, however, magnified the difference in erythropoietic activity between the extracts of the stimulated uremic plasma and the extracts of plasma from the non-stimulated, nephrectomized rabbit. This result would appear to constitute further evidence for the presence of erythropoietin in the plasma of the hypoxic, nephrectomized rabbit. The attempts to inactivate erythropoietin by uremic plasma were unsuccessful. Erslev[10] also was unable to inactivate erythropoietic factor by dialysis against uremic plasma.

An intact kidney and normal renal function seem to be essential for normal erythropoiesis. Results of experiments under way in this laboratory[3] suggest that alterations in the humoral mechanism may participate in the genesis of the anemia or chronic renal insufficiency. However, the intricacies of the relationship of the kidney to erythropoiesis remain to be defined. As shown in this article, the kidney of the rabbit cannot be the sole source of erythropoietin.

SUMMARY

Experiments were designed to measure and compare the erythropoietin content of plasma from normal and uremic rabbits with and without hypoxic or anemic stimulation. No difference in erythropoietin content could be demonstrated in the plasma of normal and of uremic animals prior to stimulation. Hypoxic and anemic stimulation increased the erythropoietin content of the plasma of both normal and uremic animals; however, the increase was less in both nephrectomized rabbits and rabbits with bilateral ureteral ligation. No erythropoietin inhibitor in uremic plasma was demonstrated by in vitro experiments. It is concluded that rabbits can increase plasma erythropoietin levels in the absence of the kidney.

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HEMOLYSATE PROTHROMBIN CONSUMPTION TIME: A NEW TEST FOR THROMBOPLASTINOGENIC COAGULATION DEFECTS

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When hemolysate, an extract of erythrocytes which contains a unique clotting factor, erythrocytin, is added to platelet-poor normal human plasma exposed only to silicone surface, good consumption of prothrombin occurs. No utilization of prothrombin results with platelet extract unless a small amount of thrombin is added or the plasma is exposed to glass. This suggests that the first step in coagulation is the activation of a plasma constituent (contact factor) probably by thrombin, its reaction with platelets to form erythrocytin, and the reaction of erythrocytin with other plasma factors to form intrinsic thromboplastin. A new test, the hemolysate prothrombin consumption time, is based on these principles. If the consumption of prothrombin is low when whole blood is clotted in glass but normal with the new test, the defect is localized in the initial stage of coagulation as exemplified by thrombocytopenia. A low consumption of prothrombin is obtained when the various plasma factors participating in the generation of intrinsic thromboplastin are deficient. The test is sensitive in detecting mild hemophilias A and B, plasma thromboplastin antecedent deficiency, pseudohemophilia B, and Hageman trait. In all, the addition of aged human serum with hemolysate normalizes the test with the notable exception of hemophilia A.

THE one stage prothrombin time is recognized as a sensitive measure for establishing the normality of the prothrombin complex which includes prothrombin, labile factor (V), stable factor (VII), and the Stuart-Prower factor. Attempts to make the prothrombin consumption test in a similar manner an over-all measure of the thromboplastinogenic factors (platelets, thromboplastinogen [VIII], and plasma thromboplastin component [IX]) have been disappointing because the test often gives normal results in mild hemophilias A and B.[1, 2] The discovery that an extract of erythrocytes, when added to blood or native plasma, causes an extraordinarily high consumption of prothrombin[3] has led to the development of a modified prothrombin consumption test which has been found to be a sensitive measure of the thromboplastinogenic activity of the blood. In this article the details of the procedure are described, its theoretic aspects discussed, and the results from its application to various hemorrhagic conditions are presented.

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METHODS

Preparation of Platelet-Poor Native Plasma.—Blood was collected by venipuncture with a silicone-coated syringe and needle and immediately transferred to thick-walled test tubes similarly coated. The tubes were chilled in an ice bath for 20 minutes and then centrifuged for 30 minutes at 12,000 r.p.m. in a Servall superspeed centrifuge kept in a cold room at 4° to 6° C.

Hemolysate Reagent.—The reagent was prepared from human erythrocytes from which platelets and leukocytes had been removed by repeated washing and differential centrifugation, as previously described.[4] A purified product with a phosphorus to nitrogen ratio of 1 to 1 prepared from the stroma of the erythrocytes was found to act similarly to hemolysate.[5]

Aged Normal Serum.—Freshly drawn human blood was transferred to clean dry glass test tubes which were put in a water bath at 37° C. Fifteen minutes after a solid clot formed, the tubes were centrifuged and reincubated at 37° C. for 1 hour. The serum was removed from the clot, placed in a refrigerator at 4° C. for 24 hours, and then stored at −20° C.

Platelet Extract.—Washed human platelets freed completely from erythrocytes were suspended in distilled water and were repeatedly frozen and thawed to disrupt the cells. The details have been described previously.[6] The platelet extract was approximately 20 times more concentrated than the platelets in blood.

Prothrombin Consumption Test (Basic).—One milliliter of freshly drawn blood or 1 ml. of plasma was transferred to a glass test tube (13 by 100 mm.) which was placed in a water bath at 37° C. Fifteen minutes after a solid clot formed, the tube was centrifuged for 1 minute. For plasma the fibrin clot was wrapped about a wooden applicator instead of being centrifuged. After 45 minutes of additional incubation at 37° C., the prothrombin remaining in the serum was determined.

Hemolysate Prothrombin Consumption Test.—One milliliter of native platelet-poor plasma was transferred to a silicone-coated test tube and 0.1 ml. of hemolyzate reagent was added. After mixing, the tube was incubated at 37° C. The clot was wrapped about a wooden applicator 15 minutes after it was formed. The serum was incubated at 37° C. for 45 minutes before the residual prothrombin was determined.

Hemolysate Aged Serum Prothrombin Consumption Test.—The procedure is the same as the preceding one except that 0.03 ml. of aged serum was added with the hemolysate.

Determination of Serum Prothrombin Time.—The principle of the test is the same as that of the one stage prothrombin time. Since the serum lacks fibrinogen and is depleted of labile factor, these have to be supplied. Freshly oxalated rabbit plasma adsorbed with $Ca_3(PO_4)_2$ to remove prothrombin and stable factor is very satisfactory because it contains a high excess of labile factor. Human plasma is less satisfactory because of its much lower concentration of this factor. Purified fibrinogen in unsatisfactory.

One tenth of the serum to be analyzed is added to a mixture containing 0.1 ml. of deprothrombinized rabbit plasma, 0.1 ml. of thromboplastin reagent and 0.1 ml. of 0.02M CaCl. . The serum must be added very rapidly. This is best accomplished either by blowing it from a pipette by mouth or by means of a rubber bulb from a medicine dropper attached to the pipette. The details of the test have been described[4]; all other tests employed were carried out as previously outlined.[4]

Cases.—The hemolysate prothrombin consumption procedure was employed in studying various hemorrhagic diseases in which the one stage prothrombin time is normal. Although the findings from only one patient for each bleeding state are reported, studies were carried out on several subjects representing each disease except Hageman trait. Only one individual with the latter condition was available. In order to show the sensitivity of the test, the results of the mildest cases available are reported.

RESULTS

Normal Blood.—Platelet-poor plasma obtained from a normal subject has a prothrombin consumption time of 12 seconds when clotted in a silicone-coated test tube and 8 to 9 seconds when clotted in glass (Table I). This shortening has been explained by the conversion of inactive prothrombin to the active state.[7] The validity of this assumption does not enter directly into consideration of the present problem. It is important, however, to recognize that clotting in silicone is different from that in glass. The addition of platelet extract to native plasma exposed only to a silicone surface will not cause any significant consumption of prothrombin[6] but when added to plasma exposed to glass will bring about good consumption. Exposure of plasma to glass is necessary for platelets to become active in the prothrombin consumption reaction.

TABLE I. EFFECT OF SURFACE ON PROTHROMBIN CONSUMPTION TIME OF PLATELET-POOR PLASMA ON ADDITION OF PLATELET EXTRACT AND ERYTHROCYTE EXTRACT

	PROTHROMBIN CONSUMPTION TIME	
	SILICONE (SEC.)	GLASS (SEC.)
Normal plasma platelet-poor	12	8
+ platelet extract	12	30
+ hemolysate	28	45
+ hemolysate and serum	50	77

Hemolysate, an extract of erythrocytes, has a high concentration of a clotting factor, erythrocytin, which when added to plasma unexposed to glass brings about a good consumption of prothrombin. This clearly suggests that its activity is independent of the activation of a plasma contact factor. The new test, the hemolysate prothrombin consumption time, is based on this observation.

Thrombocytopenia.—In thrombocytopenia, a poor consumption of prothrombin occurs which is due in the uncomplicated cases solely to lack of platelets. It is to be expected that the hemolysate prothrombin consumption time should be normal since hemolysate fully substitutes for platelet activity. The patient who had acute thrombocytopenic purpura had a completely normal consumption with the new test (Table II).

Hemophilia.—In severe hemophilia the prothrombin consumption time is 8 to 9 seconds when clotting is carried out in a glass test tube. The addition of either hemolysate or hemolysate with aged serum does not increase the prothrombin consumption. If the test is carried out in a silicone-coated test tube, a value of 11 to 12 seconds is obtained on plasma or on blood from an individual with severe hemophilia. Neither the addition of hemolysate nor hemolysate with aged serum brings about any correction. In the mild cases, hemolysate alone will not increase prothrombin consumption in a silicone-coated test tube, as seen in Table II, but if serum is added with the hemolysate, the time is increased but much less than for normal plasma. The hemolysate test carried out in a silicone-coated test tube has been found to be a more sensitive means to detect very mild hemophilia than the original prothrombin consumption test.

Hemophilia B.—The basic prothrombin consumption test carried out in glass yields abnormally short values, but even in severe hemophilia B the serum prothrombin time is never 8 to 9 seconds but always somewhat higher, as seen in Table II. No adequate explanation for this can be given. Even in mild hemophilia B, the hemolysate test in silicone yields low prothrombin consumption time values, thus making the test equally as useful to detect mild hemophilia B as it does classic hemophilia. In hemophilia B the results are sometimes erratic. Thus, in the mild case reported in Table II, the hemolysate prothrombin consumption test in a silicone tube was 8 seconds.

By adding normal aged serum with hemolysate, a prothrombin consumption time as high as for normal plasma is obtained. This serves as a simple but definitive means to differentiate hemophilia B and classic hemophilia. Serum from a patient with hemophilia B fails to correct the prothrombin consumption time.

Plasma Thromboplastin Antecedent Deficiency.—Although plasma thromboplastin antecedent (PTA) deficiency is still not clearly defined, enough cases have been reported to make a tentative diagnosis of such a condition reasonably certain. The cases studied in the present series resemble those originally described by Rosenthal and associates[8] and later by others.[9] The case recorded in Table II was selected because the patient may be regarded as a mild bleeder and because all the laboratory tests, with the exception of the new hemolysate prothrombin consumption test, had normal results. Despite the apparently mild bleeding state, she bled so severely into the soft tissues of her mouth and throat after an oral surgical operation that a tracheotomy became imperative.

Hageman Trait.—The subject who has this abnormality resembles the original cases described by Ratnoff and Colopy.[10] The prolonged clotting time was discovered by accident. He has never had abnormal bleeding although he bruises a little more than normal. A low consumption of prothrombin was found with the new hemolysate test. As in plasma thromboplastin antecedent deficiency, the addition of aged serum with the hemolysate brought about complete correction. These observations clearly suggest that the Hageman defect is not involved in the initial stage of coagulation and that, therefore, the contact factor and the Hageman factor are separate entities.

Pseudohemophilia A (von Willebrand's Disease).—The basic defect in pseudohemophilia A remains uncertain. It is now generally agreed that the platelets are normal in this disease. The prolonged bleeding time which is the most characteristic finding suggests that the defect is vascular, but the therapeutic effectiveness of a fresh plasma transfusion strongly suggests that a plasma factor is involved in the hemostatic defect. The patient whose laboratory findings are recorded in Table II has the typical hereditary pattern of an autosomal dominant trait and the bleeding history which is characteristic of this disease. The hemolysate prothrombin consumption test gave normal results.

Pseudohemophilia B.—The condition is frequently called vascular hemophilia because the bleeding time is prolonged and the concentration of thromboplastinogen (factor VIII) in the blood is low. Since the vascular abnormality may be the result of a plasma factor, the term pseudohemophilia B, proposed by Singer and Ramot,[11] seems to be preferable. The patient whose laboratory findings are recorded in Table II may be regarded as a satisfactory representative of those with the disease. She inherited the defect as an autosomal recessive trait. One of her brothers has the same disease whereas another brother and her parents are clinically normal. She has a normal clotting time but a mildly defective prothrombin consumption time and a defect in the plasma as measured by the thromboplastin generation test of Biggs and Douglas.[12] It is to be noted that the hemolysate prothrombin consumption is low but not as striking as in mild hemophilia and that the addition of aged serum with the hemolysate does not bring it to normal.

TABLE II. APPLICATIONS OF HEMOLYSATE PROTHROMBIN CONSUMPTION TEST TO VARIOUS HEMORRHAGIC DISEASES

	PROTHROMBIN CONSUMPTION TIME			
	SILICONE		GLASS	
DIAGNOSIS	HEMOLYSATE (SEC.)	HEMOLYSATE + AGED SERUM (SEC.)	HEMOLYSATE (SEC.)	HEMOLYSATE + AGED SERUM (SEC.)
1. Normal	> 25	> 45	> 40	> 45
2. Thrombocytopenia	26	81	44	70
3. Hemophilia A, mild	12	25	40	48
4. Hemophilia A, severe	*	11½	8	8
5. Hemophilia B, mild	8	47	17	55
6. Hemophilia B, severe	12	60	12	73
7. PTA deficiency	12	44	13	59
8. Hageman trait	12	48	19	48
9. Pseudohemophilia A	33	55	40	73
10. Pseudohemophilia B	15	32	20	34

*No clotting occurs.

Subject 2: Subject 2 was a boy born in August, 1954. He was well until June, 1959. At this time the parents noted petechiae and easy bruising. Laboratory studies confirmed the diagnosis of thrombocytopenic purpura. Treatment with corticosteroids for 6 months failed, but a complete recovery was brought about by splenectomy. Laboratory findings were: clotting time, 6 minutes; bleeding time, 4 minutes; prothrombin consumption time, 8 seconds; prothrombin time, 12 seconds; tourniquet test, positive; platelet count, 15,000; clot retraction, none. Diagnosis was acquired thrombocytopenic purpura.

Subject 3: Subject 3 was a boy born in August, 1954. He bled after circumcision and required a transfusion. He had a large hematoma on his forehead after a fall and once bled profusely after crushing his finger. He has had no epistaxis. On examination, one bruise on the leg was noted. All joints were found normal. Laboratory results were: clotting time, 11½ minutes; bleeding time, 2 minutes; prothrombin consumption time, 15 seconds; prothrombin time, 12 seconds; thromboplastinogen assay, 3 per cent. Diagnosis was mild hemophilia A.

Subject 4: Subject 4 was a man aged 35 years. He has been a severe bleeder since early childhood. He has had many hemarthroses and several attacks of hematuria. His maternal uncle also has severe hemophilia. Laboratory findings were: clotting time, 63 minutes; bleeding time, 3 minutes; prothrombin consumption time, 8 seconds; prothrombin time, 12 seconds. Diagnosis was severe hemophilia A.

Subject 5: Subject 5 was a boy born in 1947. He bled after circumcision. Easy bruising was noted after he began to walk. A diagnosis of hemophilia was made at the age of one year. He was given transfusions prophylactically for several months. He began to have hemarthroses early, involving the knees and ankles mainly. He has ankylosis of the right knee. He has had a retroperitoneal hemorrhage, hematuria, and intramuscular bleeding, but no epistaxis. He has not required hospitalization for the past several years. Family history: mother's maternal cousin died of hemorrhage after tonsillectomy. Laboratory findings were: clotting time, 8 minutes (has been as long as 14 minutes); bleeding time, 2 minutes; tourniquet test, normal; prothrombin consumption time, 12 seconds; prothrombin time, 12 seconds; thromboplastinogen assay, 100 per cent; thromboplastin generation test with patient's plasma, 9 seconds—with patient's serum, 24 seconds. Diagnosis was mild hemophilia B.

Subject 6: Subject 6 was a man born in 1932. He always bruised easily after slight injuries. After a fall with resultant head injury, he developed convulsions but recovered without sequellae. During childhood he was hospitalized about eight times for nosebleeds, which were caused by head colds. He bled after loss of two deciduous teeth. Although he has had numerous hemarthroses, his joints are grossly normal with no limitation of motion. He has had one attack of hematuria. He continues to bruise moderately, has no nosebleeds, and only occasionally has bleeding into his joints. He has not been hospitalized for the past 10 years. Laboratory findings were: clotting time, 30 minutes (has been more than 60 minutes on occasion); bleeding time, 3 minutes; tourniquet test, negative; prothrombin consumption time, 10½ seconds. Diagnosis was severe hemophilia B.

Subject 7: Subject 7 was a woman born in 1939. Easy bruising was noted in early childhood. She bled severely after tonsillectomy and after tooth extractions. After the removal of the submaxillary gland because of calculus which could not be dislodged, she bled into the soft tissue of the mouth and throat. A tracheotomy had to be performed. Three transfusions were required to control bleeding. She continues to bruise easily but has no other types of bleeding and has normal menstruation. Family history is negative. Laboratory findings were: clotting time, 8 minutes; bleeding time, 3 minutes; tourniquet test, negative; prothrombin consumption time, 22 seconds; prothrombin time, 12 seconds; thromboplastin generation test with patient's plasma, 13 seconds—with patient's serum 9 seconds—with patient's plasma and serum, 13 seconds. Diagnosis was plasma thromboplastin antecedent deficiency.

Subject 8: Subject 8 was a normal healthy man born in 1927. He has never had abnormal bleeding but is under the impression that he bruises more easily than is normal. The prolonged clotting time was discovered accidentally. Laboratory findings were: clotting time, from 20 to 30 minutes; bleeding time, 3 minutes; tourniquet test, normal; platelet count, 267,000; prothrombin consumption, 19 seconds (was 12 seconds on one occasion); prothrombin time, 12 seconds; thromboplastinogen assay, 44 per cent; thromboplastin generation test with patient's plasma, 9 seconds—with patient's serum, 9½ seconds—with patient's plasma and serum, 20 seconds. Diagnosis was Hageman trait.

Subject 9: Subject 9 was a woman born in 1925. She had frequent epistaxes as a child, but rarely after adolescence. She bled profusely during her first menstrual period; she required hospitalization and several transfusions. She has eleven children. She bled severely postpartum and required transfusions with five of the children. With the others, no abnormal postpartum bleeding occurred. She had no bleeding after the extraction of a third molar. One of her sisters died from postpartum hemorrhage. Her father and one paternal aunt have frequent epistaxes. Six of her children bruise more easily than normal and have epistaxes. Laboratory findings were: clotting time, 6 minutes; bleeding time, 8 minutes; tourniquet test, positive; prothrombin consumption time, 22 seconds; prothrombin time, 12 seconds; platelet count, 357,000. Diagnosis was pseudohemophilia A (von Willebrand's disease).

Subject 10: Subject 10 was a girl born in 1947. Although she bruised easily in early infancy, she had no serious bleeding until she was 18 months old when she had an apparently spontaneous cerebral hemorrhage. Although she was in a coma and later paralyzed, she made a complete recovery. She has had almost every conceivable type of bleeding, including intramuscular and joint bleeding, hematuria, intestinal hemorrhage, and epistaxis. The latter has been the most troublesome. Even though she has had over 200 transfusions, she looks healthy, is well nourished, has no joint deformities, and leads a remarkably normal life; she has a brother who has the same condition. Another brother and her parents have no bleeding tendency. The family history is entirely negative for abnormal bleeding. A more complete case history has been presented previously.[13] Laboratory findings were: clotting time, 9½ minutes; bleeding time, > 30 minutes; tourniquet test, markedly positive; prothrombin consumption time, 12 seconds (has varied from 10 to 19 seconds); prothrombin time, 12 seconds; platelet count, 390,000; thromboplastinogen assay, 1 per cent; thromboplastin generation test, with the patient's plasma, 42 seconds—with patient's serum, 11 seconds. Diagnosis was pseudohemophilia B.

DISCUSSION

The generation of intrinsic thromboplastin is brought about by a series of chemical reactions which are still not fully understood. It seems fairly clear that the initial reaction that occurs when blood clots in a test tube is the activation of a plasma constituent, which is commonly called the contact factor because its activation can be effected by exposure to a surface such as glass. The actual activator is probably not glass itself but rather thrombin which is adsorbed to its surface. When a concentrated extract of platelets is added to plasma unexposed to any surface other than silicone, no detectable consumption of prothrombin occurs. If, however, the plasma is momentarily exposed to glass or mixed with a minute amount of thrombin,[14] good consumption of prothrombin results though the coagulation takes place in a silicone-coated tube.

From this observation, it can be postulated that, after the contact factor becomes activated, it reacts with a platelet constituent. After this reaction, the clotting can go to completion without further contact with glass, as shown by the good consumption of prothrombin. Significantly, an extract of erythrocytes, when added to platelet-poor plasma which has been in contact only with silicone surface, brings about a high consumption of prothrombin, thus showing that unlike the platelet extract it does not require the contact factor. In a recent article the author[15] has postulated that erythrocytin, the active principle in hemolysate, is the product formed by the interaction of the contact factor and platelets, and that it, rather than the platelet factor, is the agent which actually participates in the generation of intrinsic thromboplastin.

The usual range of the prothrombin consumption time for normal blood clotted in glass under rigidly standardized conditions is about 16 to 35 seconds. If hemolysate is added to the blood or plasma before clotting, the prothrombin consumption time is considerably prolonged. The average range is 45 to 90 seconds. Such a striking increment is not obtained when an excess of a concentrated platelet extract is added, thus indicating that the platelets are not the limiting factor in the prothrombin consumption of normal blood. Only

when the platelet count is significantly reduced, usually to less than 50,000, is the consumption of prothrombin affected. As the platelet count is reduced below this critical level, the prothrombin consumption time becomes proportionately shortened until the minimum value of 8 to 9 seconds is reached.

It appears fairly evident that the limiting factor in the consumption of prothrombin when normal blood is clotted in a test tube is either the concentration of the contact factor or its activation; therefore, the conditions for activating the contact factor must be optimal in the basic prothrombin consumption test. Thrombin is essential and since this agent is effectively removed by adsorption to fibrin surface, little consumption can occur in the nonretracted clot with its delicate fibrin network. For this reason, 15 minutes after the clot has formed, the clotted blood is centrifuged in order to break the intimate contact of serum with fibrin surface. The accumulation of sufficient thrombin is thereby assured to catalyze the reaction between the contact factor and platelets, which is essential before platelets can enter into the reaction to form intrinsic thromboplastin.

Thromboplastinogen (factor VIII) is a primary agent in the generation of thromboplastin but it does not become a limiting factor in the prothrombin consumption test until the concentration drops approximately to 1 per cent of normal. Since it is at about this level that the bleeding tendency in hemophilia manifests itself, the prothrombin consumption test is often normal in very mild cases. When the concentration of thromboplastinogen is less than 1 per cent, the prothrombin consumption time is abnormally short and at about 0.1 per cent reaches the minimal value of 8 to 9 seconds.

Plasma thromboplastin component, which is likewise a primary agent in the generation of thromboplastin, presents a more complex problem, because this agent is in an inactive state in the blood.[2] It cannot be measured in plasma but only in serum since it is only in the latter that it is present in the active state. In its activation, plasma thromboplastin antecedent and perhaps also the Hageman factor appear to be essential. As the concentration of plasma thromboplastin component becomes progressively decreased, a level is reached at which the consumption of prothrombin falls below normal. This concentration appears to be about the critical level for hemostasis. In mild hemophilia B the prothrombin consumption time may be normal, but in the more severe cases the test is unequivocally abnormal. In mild hemophilia B the hemolysate prothrombin consumption test appears to be more sensitive than the basic prothrombin consumption test.

When the prothrombin consumption time is 15 seconds or shorter in a patient with a bleeding history, it can be concluded that faulty production of intrinsic thromboplastin is the most likely cause. It is not difficult to determine whether the defect is due to lack of platelets, thromboplastinogen, or plasma thromboplastin component. This can be done either by the thromboplastin generation test of Biggs and Douglas or the prothrombin consumption test.

The most serious shortcoming of the simple unmodified prothrombin consumption test is its relative insensitivity to the clotting defect in mild hemophilias A and B. The new hemolysate prothrombin consumption test corrects this limitation of the basic method and, therefore, effectively complements the primary procedure. In the new test, the influence of platelets and of the contact factor are eliminated and therefore normal results are obtained in severe thrombocytopenia. Significantly, very low results are obtained even in mild hemophilias A and B and in Hageman trait as well as in the condition which is commonly designated as plasma thromboplastin antecedent deficiency. The faulty prothrombin consumption is corrected in all of the latter conditions, except hemophilia A, by adding normal aged serum with the hemolysate.

The hemolysate prothrombin consumption test is not intended to replace the basic prothrombin consumption test but to complement it. In moderately severe hemophilias A and B, the new test is not needed. Its value is in the detection of mild hemophilia and of other conditions such as mild plasma thromboplastin antecedent deficiency in which other tests including that of Biggs and Douglas may give equivocal results.

The observation that the hemolysate prothrombin consumption test is normal in pseudohemophilia A or von Willebrand's disease indicates that the production of intrinsic thromboplastin is normal. Further confirmation for this conclusion is the normal thromboplastin generation as measured by the method of Biggs and Douglas which my associates and I have found in a fairly large number of cases. In pseudohemophilia B, the prothrombin consumption test is abnormal as is the thromboplastin generation test. The latter disease resembles mild hemophilia in some respects, whereas pseudohemophilia A has no resemblance to classic hemophilia or to the B type of hemophilia.

SUMMARY

A new test, the hemolysate prothrombin consumption time, has been developed. It consists in adding a standardized extract of erythrocytes (hemolysate) to platelet-poor human plasma that has been in contact only with silicone-coated surfaces, clotting the mixture at 37° C. in a silicone-coated tube, and determining the amount of residual prothrombin in the resulting serum. Good consumption of prothrombin occurs in plasma from normal subjects and patients with thrombocytopenia and pseudohemophilia A (von Willebrand's disease). The consumption of prothrombin with the new test is low even in mild hemophilias A and B, in plasma thromboplastin antecedent deficiency, in pseudohemophilia B, and in subjects with Hageman trait.

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LABORATORY METHODS

A SIMPLE METHOD FOR DETERMINATION OF TOTAL LIPIDS IN SERUM

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A simple method for the determination of total lipids in blood is presented. Based on the Schain test for butterfat, it employs detergents in the form of a single reagent to release the lipids from serum and a Babcock skimmed milk bottle in which the entire test is performed and the total lipids are measured. Compared with Bragdon's modification of Bloor's method and Sperry and Brand's procedure on random samples, the new method was in reasonably good agreement. Recoveries with the new method achieved more accurate results than did the other two methods. The total time for the new test, run in duplicate, is 30 minutes of which only 3 minutes is actual working time. It is accurate within the range of ±50 mg. per 100 ml. of blood.

RELATIVELY few methods for the determination of total lipids in serum have appeared in the literature since Bang[1] developed a procedure for oxidizing the lipids and titrating the excess of dichromate iodometrically with sodium thiosulfate, and Bloor[2] developed the method whereby a quantitative oxidation was obtained. Bloor[3] further modified the method to a colorimetric procedure which Bragdon[4] employed in modified form. Several gravimetric procedures have been proposed[5-7] but in none of these have unmodified serum lipids been determined. Each may be criticized on a procedural basis and in each the possibility exists that varying amounts of nonlipid materials may be carried through the purification process. The method of Sperry and Brand[8] combines gravimetric and purification procedures in the absence of auto-oxidation. Bloor's method or one of its modifications has been the method of choice in most clinical laboratories in which total lipid determinations are performed.

The methods now in use involve, either all or in part, extractions, evaporations, filtrations, purifications, colorimetry, and weighing. Furthermore, they may rely upon determinations such as cholesterol and lipid phosphorus, the use of tables, standard graphs, formulas, theoretic factors, and the assumption of molecular weights of complex mixtures. For the average routine clinical biochemical laboratory, present methods are prohibitive as expensive, tedious, and time consuming. A new practical test for serum lipids, based on the Schain test for butterfat,[9] is described. The procedure involves the use of a detergent to release the lipids and the use of a Babcock skimmed milk bottle for the measurement of the lipid materials.

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MATERIALS AND METHODS

Materials.—Human serum and Lipomul* were used as sources of lipid materials.

Methods.—The Schain test† is performed as follows:

Pipette 2 ml. of serum and 2 ml. of normal saline into a Babcock skimmed milk bottle.

Gently mix the serum and saline and carefully underlay 20 ml. of reagent to float the serum mixture. The Schain test reagent consists of a saline solution of alkyl phenyl polyethylene glycol ether (nonionic detergent) in a delicate balance with dioctyl sodium phosphate (anionic detergent). The nonionic detergent releases the lipids quantitatively from the serum and the anionic detergent removes the interfering proteins. The reagent also contains oil red 0 to color the released lipids and provide ease in reading.

Allow the bottle to stand at room temperature for 10 to 15 minutes, then place in boiling water for 10 minutes and in room temperature water for 2 minutes.

Add a solution of normal saline that contains 10 per cent methyl alcohol to raise the lipid material into the calibrated neck of the bottle.

Hand whirl the bottle 25 times in a centrifugal motion and read the number of divisions occupied by the lipid. The test should be performed in duplicate; readings should agree to within two divisions.

Multiply the reading by 35 to obtain milligrams per cent total lipids.

RESULTS

Calibration of Babcock Skimmed Milk Bottles Using Schain Test.—To determine the value of one scale unit on the Babcock bottle, weighed increments of Lipomul were added to a sample of human sera and total lipids were determined. The value derived was 35 mg. per cent of vegetable lipid for each scale unit (Table I).

TABLE I. CALIBRATION OF BABCOCK BOTTLES USING SCHAIN TEST ON MIXTURE OF HUMAN AND VEGETABLE LIPIDS

% LIPOMUL IN MIXTURE	ADDED VEGETABLE LIPID (MG. %)	TEST READINGS (SCALE UNITS)	UNITS ACCOUNTED FOR DUE TO ADDED VEGETABLE LIPID (SCALE UNITS)	VEGETABLE LIPID/ SCALE UNIT (MG. %)	CALCULATED VALUE OF MIXTURE* (MG. %)	TEST VALUE OF MIXTURE (MG. %)
0	0	22.25	0			779
1	142	25.75	3.5	40.6	921	900
2	284	30.00	7.75	36.6	1,063	1,050
3	426	36.00	13.75	31.0	1,205	1,260
4	568	40.00	17.75	32.0	1,347	1,400
5	710	41.00	19.25	36.9	1,489	1,453

*Based on result of this experiment giving average milligrams per cent per scale unit = 35.4.

To further test the validity of the derived value of 35 mg. per cent and to determine the range of lipid for which the Schain test is applicable, a series of 20 specimens were prepared from pooled human sera and Lipomul and were tested for total lipids by the Schain test. The increments of serum and Lipomul were taken volumetrically in arithmetic progression so calculated as to contain 50 mg. per cent difference in total lipids between specimens. This series further demonstrated the validity of 35 mg. per cent per scale unit and further identified its applicability throughout the range tested (Table II). Reproducibility and linearity are shown. Results did not vary appreciably more than ±50 mg. per cent from the calculated value.

*A product of The Upjohn Company, Kalamazoo, Mich., containing 15 per cent vegetable oil.

†Method and reagent were obtained from Merck & Co., Inc., Rahway, N. J.

TABLE II. TOTAL LIPID DETERMINATIONS BY SCHAIN TEST ON MIXTURES OF POOLED NORMAL HUMAN SERUM AND LIPOMUL

SPECIMEN NO.	DUPLICATE READINGS (SCALE UNITS)		AVERAGE READING (SCALE UNITS)	TOTAL LIPIDS		DEVIATION (MG. %)
				CALCULATED (MG. %)	DETERMINED* (MG. %)	
Human serum	21.25	20.0	20.63	750	722	-28
1	24.0	22.0	23.0	800	805	+ 5
2	23.5	25.5	24.5	850	857	+ 7
3	24.0	26.0	25.0	900	875	-25
4	27.0	26.0	26.5	950	928	-22
5	29.0	29.5	29.13	1,000	1,020	+20
6	29.5	30.0	29.75	1,050	1,041	- 9
7	31.5	33.5	32.5	1,100	1,138	+38
8	33.0	33.25	33.13	1,150	1,159	+ 9
9	34.0	36.0	35.00	1,200	1,225	+25
10	36.5	36.0	36.25	1,250	1,268	+18
11	38.0	38.0	38.0	1,300	1,330	+30
12	39.75	39.75	39.75	1,350	1,391	+41
13	38.5	40.5	39.5	1,400	1,383	-17
14	40.5	41.0	40.75	1,450	1,426	-24
15	41.5	42.0	41.75	1,500	1,461	-39
16	43.0	44.0	43.5	1,550	1,523	-27
17	45.75	45.5	45.68	1,600	1,599	- 1
18	46.5	47.0	46.75	1,650	1,636	-14
19	47.5	48.5	48.0	1,700	1,680	-20
20	50.5	50.5	50.5	1,750	1,768	+18

Total milligrams per cent difference in specimens due to added Lipomul—10,500 mg. per cent.

Total scale units difference in specimens due to added Lipomul—298.5 scale units.

*Average value per scale unit—35.2 mg. per cent.

Comparison of Schain Test and Bragdon's Colorimetric Determination for Serum Total Lipids.—The value of 35 mg. per cent per scale unit found in vegetable lipids was also assumed in the Schain test for human serum lipids. To test the validity of this assumption, a comparison of the Schain test and Bragdon's colorimetric determination on total serum lipids was performed. The agreement between these methods (as shown in Table III) was reasonably good considering the status of total lipid methods.

TABLE III. COMPARISON OF SCHAIN TEST AND BRAGDON'S COLORIMETRIC DETERMINATION OF SERUM TOTAL LIPIDS

HUMAN SERUM	SCHAIN TEST (MG. %)	BRAGDON'S COLORIMETRIC DETERMINATION (MG. %)	DEVIATIONS (MG. %)
1	603	693	90
2	603	697	94
3	614	607	7
4	639	529	110
5	677	770	93
6	718	782	64
7	726	732	6
8	726	798	72
9	726	829	103
10	735	778	43
11	735	918	183
12	750	666	90
13	803	799	4
14	1,295	1,549	254
15	1,435	1,303	132
16	1,578	1,343	235
17	1 812	1.937	125

Comparison of Recoveries Using Schain Test and Bragdon's Colorimetric Determination.—A series of specimens that contained pooled human serum to which was added serum from an individual which contained high serum lipids was run simultaneously with both procedures. By accepting values obtained with both procedures for both the low and high lipid serums, calculated values for the series were determined. For clinical purposes, the two methods are in satisfactory agreement (Table IV).

TABLE IV. COMPARISON OF SCHAIN TEST AND BRAGDON'S COLORIMETRIC DETERMINATION ON HUMAN SERUM BY RECOVERIES

SPECIMEN NO.	LOW LIPID SERUM (ML.)	HIGH LIPID SERUM (ML.)	SCHAIN TEST		BRAGDON'S METHOD	
			CALCULATED VALUE (MG. %)	TEST VALUE (MG. %)	CALCULATED VALUE (MG. %)	TEST VALUE (MG. %)
1	6	0		803		799
2	0	6		2,350		2,548
3	5.75	0.25	868	858	872	807
4	5.5	0.50	932	932	945	1,046
5	5.25	0.75	995	976	1,018	1,017
6	5.0	1.0	1,061	1,033	1,091	1,064
7	4.5	1.5	1,190	1,190	1,236	1,190
8	4.0	2.0	1,319	1,300	1,382	1,258
9	3.0	3.0	1,575	1,610	1,678	1,454
10	2.75	3.25	1,641	1,654	1,746	1,640

Comparison of Schain Test and Method of Sperry and Brand.—A series of specimens was run simultaneously with both procedures. Several of the specimens contained measured amounts of Lipomul and pooled human serum so that by accepting values obtained by both procedures for the pooled human serum, calculated values were determined. For clinical purposes, the two methods are in satisfactory agreement (Table V).

TABLE V. COMPARISON OF SCHAIN TEST AND METHOD OF SPERRY AND BRAND

SPECIMEN	NORMAL HUMAN SERUM (ML.)	LIPOMUL	CALCULATED VALUE (MG. %)	SCHAIN TEST (MG. %)	METHOD OF SPERRY AND BRAND (MG. %)
A	*	—	—	662	663
B	10*	0.05	734	722	763
C	10*	0.20	944	985	938
D	10*	0.25	1,012	962	1,075
E	10*	0.5	1,345	1,295	1,517
F	†		—	682	637
G	†		—	753	719

*Pooled human serum.
†Individual serum.

DISCUSSION

Present methods for the measurement of total lipids in blood are time consuming, tedious, and require the services of a better than average technician. Duplicate determinations often vary by 10 to 20 per cent. The method under discussion, a modification of the Schain detergent test for butterfat in milk, permits the release of lipids quantitatively during the heating process. When

brought to the level of the calibrated scale of a Babcock skimmed milk bottle these lipids can be measured accurately to within ¼ of a scale division, or 8.5 mg. per cent (Table I). The test requires a total of 30 minutes for duplicate samples, of which only 3 minutes is actual working time. The apparatus required is simple, inexpensive, and readily available in most laboratories. The test, showing reproducibility, reliability, and linearity, can be run by the average technician, and yields results differing by ±50 mg. per cent for values within the total lipid range of 600 to 1,750 mg. per cent. Our experience with this test indicates that the normal human lipid range is 600 to 800 mg. per cent.

The three most important points for accurate determinations are (1) accurate pipetting of homogeneous specimens of serum, (2) heating for 10 minutes at 100° C., and (3) release of any fat particles caught in the elbow of the skimmed milk bottle into the calibrated neck of the bottle. It is extremely important that the serum specimen be evenly mixed before drawing up the 2 ml. sample since in sera with a high total lipid content there is a tendency for the lipid materials to separate. The test results are altered if the proper temperatures and heating times are not carefully followed. Lower heating temperatures or shorter heating times will result in lower readings, especially if the heat is less at the time the bottle is first placed in the water. The recommendations of having ample water at or very near 100° C. and sufficient heat available to maintain the bath at that temperature should be followed to prevent lower readings. Higher readings will be obtained by prolonged heating as a result of the presence in the detergent of a fat miscible component which with excessive heating will add on to the fat layer. These discrepancies may be avoided by heating at 100° C. for precisely 10 minutes. The structure of the skimmed milk bottle is such that small fat globules may be trapped behind the angle of the delivery tube. Each bottle should be held at an angle of 45 degrees and tapped gently with the rubber end of a pencil to release the particles. Adherence to these recommendations will result in highly accurate readings.

It is not known whether the fat extraction is complete or whether the released lipid material is completely free of nonlipid or lipid contaminants. However, the reagent gives reproducible results and recoveries, and the results, for clinical purposes, conform to those obtained with two generally accepted methods. The method is not limited by the range of only 50 calibrated units on the Babcock skimmed milk bottle (1,750 mg. per cent). It can be applied to sera with a higher total lipid content by diluting any serum yielding results above the limits of the bottle with a serum of normal total lipid content and determining the values of the normal serum and the mixture. The original concentration can be calculated as done in Table III by applying the following formula:

$$\frac{(Cm \times Vm) - (Ck \times Vk)}{Vu} = Cu$$

where C = concentration, V = volume, m = mixture of sera, k = known serum, and u = unknown serum.

The value of this test lies in the fact that it is extremely simple, making possible the use of total lipid studies in every clinical laboratory.

SUMMARY

A simple method for the determination of total lipids in blood is presented. It is based upon the release of lipids from serum by a detergent and the measurement of the released lipids volumetrically in a Babcock skimmed milk bottle. Total time for one test run in duplicate is 30 minutes, of which only 3 minutes is the actual working time. This method is accurate within ±50 mg. per 100 ml. of blood.

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METHODS OF SIMULTANEOUS MEASUREMENT OF EXCHANGEABLE SODIUM AND POTASSIUM IN MAN

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Simultaneous measurement of total exchangeable sodium (Na_e) and potassium (K_e) using Na^{24} and K^{42} can be accomplished through separation of these ions chemically, physically, or biologically. Chemical separation by techniques available to date is laborious. Using 0.3 μc per kilogram of Na^{24} and 1.5 μc per kilogram of K^{42}, it was possible to determine Na_e and K_e through measurement of combined radioactivity in a well scintillation counter with relatively high efficiency for Na^{24} and a Geiger-Müller counter with relatively high efficiency for K^{42} with use of simultaneous equations. Alternatively, measurement of exchangeable sodium and potassium could be accomplished using only a single gamma- or a single beta-sensitive isotope counter if two different biologic fluids with varying sodium to potassium ratios were used, i.e., serum and urine or serum and saliva. Finally, an optimum simultaneous method is described in which serum is counted by a gamma-sensitive method favoring Na^{24} while saliva or urine is counted by a beta-sensitive method favoring K^{42}. Accuracy to ±2 per cent is obtainable.

ISOTOPES for measurement of 24 hour exchangeable sodium (Na_e) and potassium (K_e) are now widely available. The short physical half-lives and counting characteristics of Na^{24} and K^{42} allow these measurements to be made with very little radiation exposure to the patient. Where indicated, the determinations may be repeated at short intervals, since the total body exposure assuming no excretion of either isotope is less than 0.1 r.

Simultaneous measurement of Na_e and K_e presents the problem of determination of the individual activities of Na^{24} and K^{42} at low levels of activity. Three general methods of separation have been employed; chemical separation, physical separation, and biologic separation. Chemical separation has been accomplished by precipitation of potassium salts[1] and by ion exchange resin,[2] but these methods are relatively laborious and will not be discussed in this article. Physical separation depends upon differentiation of the emission particles of Na^{24} from those of K^{42}, since the half-lives are too similar to aid in separation (Fig. 1). Biologic separation depends on the partial separation of Na^{24} and K^{42} provided by body fluids having different sodium to potassium ratios.

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In this article, simple methods of physical and biologic separation, and a method combining physical and biologic separation are presented.

MATERIALS AND METHODS

Studies were performed on twenty-one patients and twelve normal volunteers at the National Cancer Institute. None of the patients had excessive sweat or gastrointestinal electrolyte losses. Several patients had moderate wasting from malignant disease. Patients with large tumor masses were not studied because of lack of evidence that isotope equilibration with tumor sodium and potassium occurs by 24 hours. Patients with circulatory insufficiency or with edema were not studied because of the possibility that equilibration might not be complete at 24 hours.

In all studies, the following outline was used:

1. Na^{24} and K^{42} were received in solution from Oak Ridge National Laboratories Tuesday mornings. The solutions were diluted with saline, sterilized, and were ready for patient use within a few hours.
2. Patient voided just prior to injection.
3. About 0.3 μc per kilogram of Na^{24} and 1.5 μc per kilogram of K^{42} were injected from calibrated 10 ml. syringes ($\pm$0.001 ml.) into the tubing of a freely running infusion of 5 per cent dextrose solution.
4. The urine output in the 24 hours after injection was collected and pooled for measurement of isotope excretion.
5. No sodium or potassium intake was allowed later than 18 hours after injection.
6. At 25 hours after injection serum, saliva, and urine were collected for determination of the equilibration specific activities.

Saliva flow was stimulated by chewing a piece of paraffin (about the size of a piece of chewing gum). The paraffin was chewed for 10 to 15 minutes before sample collection to ensure removal of any loose debris containing sodium or potassium which might introduce errors in the specific activity of salivary sodium and potassium.

A well-type gamma scintillation counter (Nuclear DS-3) and an end window Geiger-Müller tube (Nuclear D-47) were used for radioactivity measurement. Four milliliter liquid samples in the Geiger-Müller counter gave adequate counting rates and excellent reproducibility. In the preparation of the 2 ml. samples for the Geiger-Müller counter, a thin film of silicon grease was applied to the side of the planchet and a tiny drop of detergent was added to avoid varying wetting and surface tension effects. With counting times of less than 20 minutes, evaporation was found to have negligible effects on counting rates. With planchets of 25 mm. diameter and 6 mm. depth, the sample thickness was about 4 mm. This relatively thick liquid sample acts as a shield and favors counting of the more energetic K^{42} beta. Isotonic saline was used in preparation of isotope standards to minimize internal absorption differences between standard and sample. Internal absorption differences caused by variation in specific gravities of urine or saliva were negligible. The approximate efficiencies in the well counter are 43 per cent for Na^{24} and 6 per cent for K^{42}; in the Geiger-Müller counter, 12 per cent for Na^{24} and 22 per cent for K^{42}. Each sample was counted by each method and simultaneous equations were solved for Na^{24} counts per minute and K^{42} counts per minute (see Appendix I).

All counting values were corrected to the same time, since there is approximately a 1 per cent decrease in activity every 12 minutes. If counting is completed in less than 2 hours this correction need not consider varying K^{42} and Na^{24} content of samples, and time corrections can be made using decay values for either isotope. Time corrections should be made to the mid-point of the counting period.

Doses of 0.3 μc per kilogram of Na^{24} and 1.5 μc per kilogram of K^{42} gave adequate counting rates. All samples were counted in duplicate or triplicate to at least 5,000 counts yielding a counting error of less than 1 per cent. Total body radiation was less than 0.1 r.[3]

Sodium and potassium measurements were done in duplicate by internal standard flame photometer (accuracy 1 per cent).

RESULTS AND DISCUSSION

In the isotope dilution formula used to measure Na_e (or K_e),

$$Na_e = \frac{\text{c.p.m. retained}}{SA} = \frac{\text{c.p.m. injected} - \text{c.p.m. excreted}}{\text{c.p.m./mEq.}}$$

where specific gravity (SA) is the amount of Na^{24} per milliequivalent of sodium (c.p.m./mEq.). Thus the units of Na_e and K_e are independent of anatomic volume compartments in the body. The counts per minute retained is the difference between the amount of isotope injected and the amount excreted in the urine. The specific activity may be obtained from any equilibrium sample at 24 hours.

Serum and urine have been shown to be in equilibrium (to have the same specific activity) at 24 hours.[4-6] Saliva has also been shown to be in equilibrium with serum at 24 hours,[7, 8] and Table I shows a comparison of specific activities of saliva and urine for K^{42}.

TABLE I. COMPARISON OF SALIVA AND URINE SPECIFIC ACTIVITIES AT 24 HOURS FOR K^{42}

SUBJECTS	SALIVA SPECIFIC ACTIVITY/URINE SPECIFIC ACTIVITY
Patients	
Ber.	1.027
Zir.	1.045
Ber.	0.997
Rat.	0.987
Zir.	1.027
Som.	0.996
Bor.	1.091
Som.	1.078
Normal Controls	
Sha.	0.926
Ste.	0.953
Bor.	0.982
Whi.	1.034
Sha.	0.985
Cam.	1.027
Wms.	0.916
Ste.	1.091
Win.	1.005
Mean	1.005
Standard deviation	0.043

Isotope Excretion.—In order to measure isotope retained accurately, the isotope excretion must be known. Extrarenal excretion is negligible in patients without excessive gastrointestinal and sweat losses.[2, 4] Both Na^{24} and K^{42} excretion were easily and accurately obtained without isotope separation. The urinary Na^{24} excretion (0 to 24 hours) was calculated from the total 0 to 24 hour sodium excretion and the equilibrium specific activity, since the ratio (Table II) of the 0 to 24 hour urine specific activity to the 24 hour specific activity is 1.06* (standard deviation 0.04). Thus, Na^{24} excretion equals 1.06 × (24

*Reflecting the rapid mixing of Na^{24} with most of the Na_e.

hour specific activity) × (0 to 24 hour urine sodium). Although this calculation of Na^{24} excreted may occasionally be in error by 5 to 10 per cent, it will rarely introduce more than 0.5 per cent error in the Na^{24} retained (Table II).

TABLE II. CALCULATON OF Na^{24} EXCRETION

SUBJECT	0-24 HR. SA/24 HR. SA	MEASURED Na^{24} RETENTION (% INJECTED DOSE)	CALCULATED Na^{24} RETENTION
Patient			
Ros.	1.08	95.7	95.8
Jen.	1.07	95.8	95.8
Ben.	1.03	97.1	97.0
Bar.	1.05	92.5	92.4
Sim.	1.03	95.8	95.7
Mar.	1.07	94.4	94.5
Mur.	1.02	99.5	99.5
Ave.	*	98.2	—
Phe.	*	99.9	—
Wei.	1.05	98.7	98.7
Ogl.	1.03	97.0	96.9
Normal Controls			
Lit.	1.03	90.6	90.3
Ful.	1.09	93.8	94.0
Hof.	1.11	93.7	94.0
Sch.	1.06	95.5	95.5
Cam.	1.06	91.6	91.6
Wil.	1.17	90.4	91.5
Win.	1.00	94.9	94.6
Mean	1.058	95.7	
Standard deviation	0.041		

*The data from these two patients are not used for specific activity comparisons because of inability to void at time of isotope injection, and a low urine counting rate not giving statistical accuracy.

TABLE III. CALCULATION OF K^{42} EXCRETION

SUBJECTS	0-24 HR. SA/ 24 HR. SA	MEASURED % K^{42} RETENTION	CALCULATED % K^{42} RETENTION	ERROR (%)
Patients				
Sha.	1.70	94.8	94.8	0.0
Ste.	1.65	93.4	93.2	0.2
Bor.	1.84	94.9	95.3	0.4
Joy.	1.18	96.2	95.0	1.2
Wil.	1.37	96.9	96.1	0.8
Ber.	1.64	97.1	97.0	0.1
Ber.	1.52	96.2	95.8	0.4
Rat.	2.25	95.2	96.7	1.5
Rob.	1.53	87.4	85.9	1.5
Normal Controls				
Wil.	1.94	95.2	95.8	0.6
Cam.	2.11	93.9	94.8	0.9
Win.	2.22	95.5	96.8	1.3
Mean	1.72	94.7		
Standard deviation	0.33			

The 0 to 24 hour urinary K^{42} can be calculated in a similar manner, but the ratio of the 0 to 24 hour specific activity to the 24 hour specific activity (about 1.70) is quite variable (standard deviation 0.3) and often introduces an error

of 1 to 2 per cent in the calculation of K^{42} retained (Table III). K^{42} excretion is more accurately obtained from total urinary counts per minute minus calculated Na^{24} counts per minute.

Physical Separation of Na^{24} and K^{42}.—Since the half-lives of Na^{24} and K^{42} are too similar to aid in separation, differences in the decay schemes of the emission particles must be used for differentiation (Fig. 1). Na^{24} has about eight times more gamma emission than K^{42} and the K^{42} beta is of very high energy. Thus two counting methods, one sensitive to gamma rays and the other favoring high energy beta particles, serve to separate Na^{24} from K^{42} in a mixed sample. Each sample is counted by both methods and simultaneous equations are solved for Na^{24} and K^{42} counts per minute (see Appendix I).

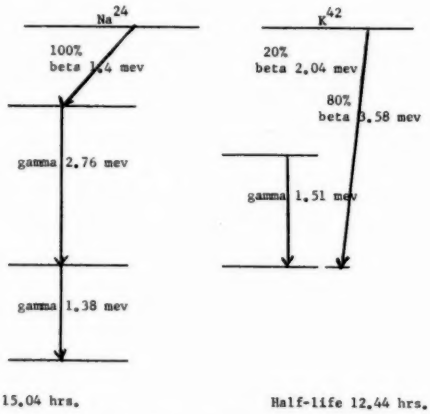

Fig. 1.—Na^{24} and K^{42} decay patterns. (From Radiologic Health Handbook, U. S. Public Health Service, Department of Health, Education, and Welfare, Washington, D. C., October, 1955, Government Printing Office.)

The ideal gamma ray–sensitive method is the well-type gamma scintillation counter. For high energy beta counting a well-type beta-sensitive phosphor,[9] a Geiger-Müller dip counter,[10] and an end window Geiger-Müller tube counter (this laboratory) have been used. The gamma-sensitive well counter can be made more specific for Na^{24} by addition of a pulse height analyzer, but this was not necessary and often lowered the observed counting rates excessively requiring additional counting time. Additional lead shielding (630 mg. per square centimeter) was used in the well counter to absorb the high energy K^{42} beta particles, thus making this counting method more specific for Na^{24} with only a slight loss of sensitivity.

When flame photometer and counting measurements are accurate to 1 per cent, the specific activities of sodium and potassium are theoretically accurate to about 1.4 per cent.

Biologic Separation of Na^{24} and K^{42}.—This method used the partial separation of Na^{24} and K^{42} provided by body fluids with different sodium to potassium ratios and has been previously described.[11] Only a single counting method is needed, and either a well-type scintillation counter or Geiger-Müller tube can be used. Proper choice of biologic samples, however, is necessary.

Serum and saliva (or urine) are especially adaptable, as they are easily collected and afford considerable separation—the serum ratio of sodium to potassium concentrations in milliequivalents per liter being about 140:4 and the saliva ratio being about 20:20. Using 1.5 μc per kilogram of K^{42} and 0.33 μc per kilogram of Na^{24}, K^{42} will predominate in the saliva while Na^{24} predominates in the serum. Under these circumstances, simultaneous determinations (see Appendix II) allow accurate determination of specific activities once the serum and saliva sodium and potassium concentrations are known.

Two to 5 ml. samples give satisfactory counting rates when either of the counting methods described is used. When flame photometer and counting measurements are accurate to 1 per cent, the specific activities of sodium and potassium are theoretically accurate to about 1.4 and 4 per cent, respectively, with the counting rates obtained in the present experiments. The Na^{24} and K^{42} excretions are obtained without isotope separation as described previously under Isotope Excretion.

Combination Physical and Biologic Separation of Na^{24} and K^{42}.—A combination of the physical and biologic separation methods provides the most practical and accurate method for simultaneous measurement of Na_e and K_e. Serum with its high sodium content is counted by the Na^{24}-sensitive method and saliva or urine is counted by the K^{42}-sensitive method. Sodium and potassium concentrations are measured in both samples.

The approximate specific activity of Na^{24} is estimated from the serum, ignoring the K^{42} activity. The specific activity is used to calculate the approximate Na^{24} counts per minute in saliva or urine, which is then subtracted from the total counts per minute giving the approximate K^{42} counts per minute and an approximate specific activity of K^{42}. This specific activity is used to correct the serum for K^{42} activity, which allows the exact specific activity of Na^{24} to be calculated. This exact specific activity of Na^{24} is then used (as before) to calculate the exact specific activity of K^{42} from saliva or urine. Specific activities by this method are theoretically accurate to about 2 per cent.

SUMMARY

Methods for simultaneous measurement of exchangeable sodium and potassium using Na^{24} and K^{42} are presented.

1. A method of isotope separation using the differential counting afforded by a well-type gamma sensitive scintillation counter and an end window Geiger-Müller tube is described.

2. A second method necessitates only one counting method and uses the biologic separation provided by body fluids (serum and saliva or urine) with different sodium to potassium ratios.

3. A combination method using both physical and biologic separation principles is presented. This affords the most efficient separation and is the most accurate method, giving a theoretic accuracy of 2 per cent for both exchangeable sodium and potassium.

The measurement of Na^{24} and K^{42} urinary excretion without isotope separation is described.

APPENDIX I

Equations for the physical separation method are presented. One sample is counted by two counting methods.

Definitions: T = total counts per minute of the sample; N = Na^{24} counts per minute; K = K^{42} counts per minute.

The subscripts 1 and 2 refer to the first and second counting methods. For the first counting method,

$$T_1 = N_1 + K_1 \tag{1}$$

For the second counting method,

$$T_2 = N_2 + K_2 \tag{2}$$

Let n and k be the ratios N_2/N_1 and K_2/K_1 (as determined from pure isotope samples). Then (2) becomes

$$T_2 = nN_1 + kK_1 \tag{3}$$

Multiply (1) by n and subtract from (3), and solve for K_1:

$$K_1 = \frac{T_2 - nT_1}{k - n} \tag{4}$$

Substituting (4) into (1) and solving for N_1 gives

$$N_1 = T_1 - \frac{T_2 - nT_1}{k-n} = \frac{kT_1 - T_2}{k - n} \tag{5}$$

Since n and k are known and T_1 and T_2 are the observed activities at a given time, K_1 and N_1 (the K^{42} and Na^{24} activity) can be calculated from (4) and (5).

APPENDIX II

Equations for the biologic separation method are presented. Two samples (i.e., serum and urine or saliva) are counted by one counting method, and the sodium and potassium concentrations must be known to allow calculation of specific activities.

Definitions: T, N, and K are defined in Appendix I; (Na) and (K) are sodium and potassium concentrations; SA^N is sodium SA, or N/(Na); SA^K is potassium SA, or K/(K). The letters s and u refer to serum and urine.

From the definitions of SA^N and SA^K,

$$N = SA^N(Na) \tag{1}$$

and

$$K = SA^K(K) \tag{2}$$

For serum,

$$T_s = N_s + K_s \tag{3}$$

Substitution of (1) and (2) for N and K in (3) gives

$$T_s = SA^N(Na)_s + SA^K(K)_s \tag{4}$$

Similarly, for urine

$$T_u = SA^N(Na)_u + SA^K(K)_u \tag{5}$$

(Note that SA^N and SA^K are the same in serum and urine because equilibrium exists.) The only unknowns in equations (4) and (5) are the desired values of SA^N and SA^K. Let $r = (Na)_u/(Na)_s$. Then multiply (4) by r and subtract from (5). Solve the resulting equation for SA^K to get

$$SA^K = \frac{T_u - r(T_s)}{(K)_u - r(K)_s} \tag{6}$$

Solving (4) for SA^N gives

$$SA^N = \frac{T_s - SA^K(K)_s}{(Na)_s} \tag{7}$$

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A SIMPLIFIED PROCEDURE FOR THE DETERMINATION OF BUTANOL EXTRACTABLE IODIDE IN SERUM OR PLASMA

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The present investigation was prompted by the lack of a simple yet accurate method for the determination of thyroxine in serum or plasma. The new method described in this article employs conventional techniques for the extraction of serum or plasma with n-butyl alcohol and for washing of the extracts with strong alkali to remove any free iodide or iodotyrosines present. After the washing, the butanol extracts are treated by shaking with aqueous ceric sulfate in acid medium to oxidatively split off the thyroxine iodine. After the aqueous-butanol layers are allowed to separate, the ceric sulfate layer is removed and the extracts are washed with distilled water. The iodine, held in the butanol layer, is reduced with sodium hydroxide and potassium bromide and extracted as iodide into the water phase. This is followed by the analysis of the iodide content by the spectrophotometric determination of the color change in the ceric sulfate–arsenious acid system. This simple method is short and accurate and no problems of contamination were encountered.

THE value of protein-bound iodine or butanol extractable iodide determinations in the clinical diagnosis of thyroid state is well known.[1] Numerous methods for the indirect determinations of blood thyroxine have been devised, all of which are based on the analysis of thyroxine iodide. Since iodide catalyzes the reaction between arsenite and cerate, minute amounts of it can be accurately measured colorimetrically after the iodide is freed from its organic binding.

Two major approaches to the problem of assaying thyroxine iodide have been employed. In the classic protein-bound iodide methods,[2] thyroxine is precipitated with the plasma proteins to which it is bound. The chemical hydrolysis of thyroxine iodide or incineration of the protein residue is followed by the colorimetric determination of the free iodide. In the butanol extractable iodide methods, the plasma or serum is first extracted with acidified *n*-butyl alcohol and the extract is then washed with strong alkali to remove any inorganic iodide or iodotyrosines present in the extract. After the washing, the butanol is evaporated to dryness and the thyroxine iodide is released either by chemical means[3] or by incineration of the residue.[5] The advantages of the butanol extractable iodide over the protein-bound iodine methods are well established.[1, 4]

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Despite the fact that the ultimate values of blood thyroxine iodide analyzed by the various methods are similar, the methods now in use are most tedious and time consuming and involve the use of cumbersome and expensive equipment. The need for an accurate but simple and short method for butanol extractable iodide prompted the present work.

MATERIALS AND METHODS

Chemical Reagents.—

1. *n*-Butyl alcohol (Mellinckrodt reagent).

2. Sulfuric acid 10 per cent volume per volume and 0.35N solutions.

3. Four normal NaOH in 5 per cent Na_2CO_3.

4. Ceric sulfate (0.1N) in 5N H_2SO_4; this reagent is prepared by dissolving 33.35 Gm. anhydrous $Ce(SO_4)_2$ in 1 L. of 5N sulfuric acid. Upon standing for a number of days a precipitate forms. The solution is filtered through a sintered glass funnel. This solution may be kept for as long as one year.

5. $Ce(SO_4)_2$ (0.005N); this reagent is used for the oxidative splitting of the thyroxine iodide. It is prepared by 1:20 dilution of reagent No. 4 with 0.35N H_2SO_4.

6. Sodium hydroxide (0.25N).

7. Potassium bromide (3 mg. per milliliter) prepared by dissolving 3 Gm. KBr in 1,000 ml. distilled water. This solution is diluted 1:100 prior to use. It may be kept for as long as one month.

8. H_2AsO_3–(0.3N); 14.84 Gm. As_2O_3 are dissolved in approximately 500 ml. of water and 28 ml. of concentrated H_2SO_4. To ease the solution of the arsenite, the mixture is heated in a water bath and shaken constantly. After cooling, the solution is made up to 1,000 ml. with water.

9. $Ce(SO_4)_2$ (0.01N); reagent No. 4 is diluted 1:10 with 10 per cent volume per volume H_2SO_4.

10. Thyroxine solution for standard curve; 100 mg. of L-thyroxine is dissolved in 1,000 ml. 0.02N NaOH. This solution is diluted 1:100 with distilled water to yield a final concentration of 1.0 meg. thyroxine per milliliter. This constitutes the working standard.

Materials.—Materials used are (1) 5 ml. glass stoppered centrifuge tubes, (2) 1 mm. stirring rods and long disposable-type pipettes, (3) Burrel "wrist action" mechanical shaker with accommodation for 16 or 24 separatory funnels, (4) constant temperature bath supplied with an electric stirrer and kept at exactly 42° C., (5) 60 ml. separatory funnels (Kontes glass) preferably supplied with Teflon stopcocks, and (6) Coleman Junior spectrophotometer and 19 by 150 mm. colorimeter tubes. All glassware is washed as for any of the protein-bound iodine or butanol extractable iodide methods. Water used for washing the glassware and for making up the reagents should contain no traces of iodide.

ANALYTIC PROCEDURE

The 60 ml. separatory funnels are secured in the Burrell mechanical shaker. The stopcocks are greased with butanol. The separatory funnels remain on the shaker throughout the entire procedure. Cylindrical funnels were found to be most effective.

1. *Butanol Extraction.*—Serum or plasma (0.5 ml.) is extracted in a 5 ml. glass stoppered centrifuge tube with 0.2 ml. 10 per cent volume per volume H_2SO_4 and 3 ml. *n*-butyl alcohol by shaking for 60 seconds.[5] After centrifugation for 15 minutes at 2,000 r.p.m., the butanol extract is carefully transferred into a 60 ml. separatory funnel (already secured to the Burrell shaker) by means of a long disposable pipette. The serum residue is extracted two more times with 0.2 ml. 10 per cent volume per volume H_2SO_4 and 2 ml. butanol by shaking for 30 seconds after the residue in the tube is broken up with a 1 mm. stirring rod. After centrifugation for 15 minutes at 2,000 r.p.m., the second and third extracts are added to the first by means of the same disposable pipette.

2. *Alkali Wash.*—To each separatory funnel, containing the pooled butanol extracts, 7 ml. 4N NaOH in 5 per cent Na_2CO_3 is added. The funnels are stoppered and mechanically shaken vigorously for 5 minutes. After standing for adequate separation of the layers (5 to 10 minutes), the alkali wash is removed. The washing procedure is repeated twice. In the third washing, however, the mixture is allowed to stand for 20 to 30 minutes for complete separation of the layers. After the bottom aqueous layer is removed, the funnels are shaken gently for a few seconds and allowed to stand for an additional 5 to 10 minutes prior to the removal of the remaining alkali wash.

3. *Oxidative Splitting of Thyroxine Iodide.*—Four milliliters 0.005N $Ce(SO_4)_2$ in 0.35N H_2SO_4 is added to the washed butanol extracts and the separatory funnels are shaken vigorously for one hour at room temperature. When the reaction in which the iodine is split from the thyroxine molecule is complete, the $Ce(SO_4)_2$ layer is removed. The butanol is washed by adding 7 ml. distilled water, shaking for 5 minutes, and draining the water layer in the exact manner in which the third alkali wash is removed.

4. *Reduction of Liberated Iodine.*—To each separatory funnel is added 1.0 ml. of 0.03 mg. per milliliter potassium bromide solution and 4 ml. 0.25N NaOH; the funnels are then shaken for 15 minutes. After 20 to 30 minutes of standing, the aqueous layers containing the iodide are drained into 19 by 150 mm. colorimeter tubes. After gentle shaking for a few seconds and standing for 5 to 10 minutes, the remaining solutions are combined with those already in the colorimeter tubes.

5. *Estimation of Iodide.*—To each colorimeter tube is added 3 ml. of 0.3N $H_2AsO_3^-$. The contents of the tube are mixed thoroughly and the tubes are placed in the constant temperature bath maintained at exactly 42° C. Also placed in the bath is an appropriate amount of 0.01N $Ce(SO_4)_2$ solution. When temperature equilibrium is attained (10 minutes) 1 ml. of the ceric sulfate solution is pipetted into each colorimeter tube at convenient time intervals. Immediately after the addition of the ceric sulfate, the contents of each tube are thoroughly mixed. At *exactly* 30 minutes after the addition of the ceric sulfate, the colors are read at 400 mμ in the spectrophotometer; the instrument is adjusted to zero optical density with water. The reaction may be stopped, if so desired, with silver nitrate as recommended by Kontaxis and Pickering.[5]

6. *Standard Curve.*—Serum or plasma (0.5 ml.) is added into each of a number of 5 ml. glass stoppered centrifuge tubes. Thyroxine standard solution is added to each tube in increasing amounts from zero to 0.12 ml. (zero to 0.12 mcg. thyroxine per tube). To a blank tube, 0.5 ml. distilled water is added. All samples are carried through the entire analytic procedure described. The optical density value for each individual sample is then divided by the blank optical density value, and the resulting optical density ratios are plotted on semilogarythmic paper against concentration of thyroxine *added* to each tube (Fig. 2, *A*). The points are connected by a straight line. The standard curve is obtained by plotting a line parallel to the line derived experimentally and intercepting the y axis at optical density ratio of 1.0. To express the concentration of iodide in any serum sample in micrograms per cent, the value read from the standard curve is multiplied by 130. If preferred, the colors in the tubes may be read in per cent transmittance. The spectrophotometer is adjusted to 100 per cent transmittance with water. The per cent transmittance of the blank is then subtracted from the per cent transmittance of each sample and the results are plotted on decimal paper against concentration of *added* thyroxine (Fig. 2, *B*). The points are connected by a straight line. The standard curve is obtained by plotting a line parallel to the experimental line and running through the origin.

RESULTS AND DISCUSSION

Extraction and Washing.—The butanol extraction and alkali washing for the removal of iodide and iodotyrosine contaminants have been adequately

worked out and discussed elsewhere.[3, 5-7] The extraction and washing procedures employed here are essentially those of Kontaxis and Pickering.[5] It was found that added iodide and iodotyrosines were removed by the alkali wash.

A thyroxine curve, run with varying amounts of the hormone added to washed butanol, is compared with a curve run with thyroxine added to serum (Fig. 1). It is seen that about 10 to 12 per cent of the serum thyroxine is lost during the extraction and the washing. This prohibits the use of standard curves based on recoveries of thyroxine from washed butanol and it is for this reason that our standard curves are established on thyroxine recoveries from serum or plasma. No differences are found between serum and plasma, and either sample can be read on either curve.

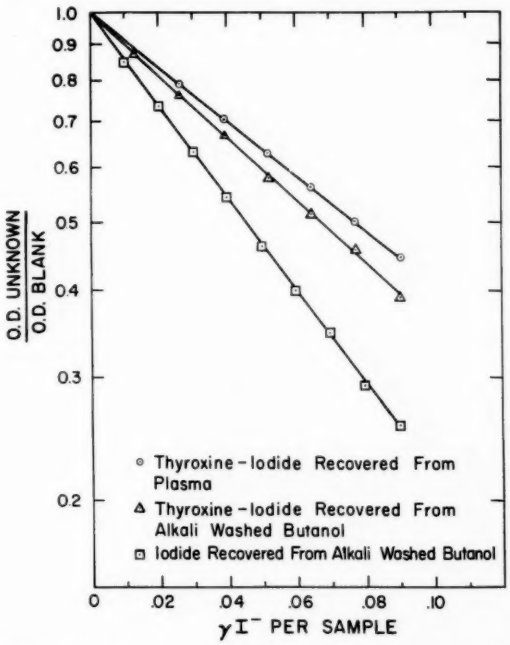


Fig. 1.—Standard curves determined with: (a) thyroxine added to plasma, (b) with thyroxine added to 7 ml. alkali washed butanol, and (c) pure iodide added to 7 ml. of alkali washed butanol.

Ceric Sulfate Oxidative Splitting of Thyroxine Iodide.—Bowden and associates[8] have shown that ceric sulfate may split the iodine off iodinated amino acids and have used a ceric sulfate-arsenite mixture to detect iodinated amino acids on paper chromatograms. The iodide splitting is apparently incomplete, as can be seen in Fig. 1 where a comparison is made between recoveries of thyroxine with recoveries of pure iodide each added to alkali washed butanol. The iodide fraction removed from the thyroxine molecule is constant, however, and enough iodide is released for purposes of detection. The reaction is carried out in an acid medium. It is very important, therefore, to remove the highly concentrated alkali wash as completely as possible.

After the bulk of the last alkali wash is removed, gentle shaking and additional standing assist in the complete separation of the two layers. The distribution coefficient for iodine between water and butanol is such that the iodine remains entirely in the butanol phase after the removal of the aqueous, ceric sulfate layer.

Reduction of Released Iodine.—During the splitting of the iodide from the thyroxine molecule, the iodide is oxidized to iodine by the ceric sulfate. As stated before, the iodine remains in the butanol layer. In order to extract the iodine from the butanol, the iodine is first reduced by shaking with potassium bromide in an alkaline medium. The exact nature of the chemical reactions involved is not known.* The reduction of the iodine and its subsequent extraction into the aqueous layer were attempted with numerous substances in both acid and alkaline media. Arsenite, thiosulfate, sulfate, chloride, and other compounds were tried. The presence of other strong reducing agents may interfere with the color reaction.

The iodide is preferentially soluble in water and is therefore extracted from the butanol when the butanol-aqueous layers are allowed to separate at the end of the reduction step.

Bromide may catalyze the arsenite-cerate redox reaction when it is present in large concentrations. However, the amounts of bromide used here are not large enough to affect the color reaction. In comparing iodide time curves with and without the addition of bromide, no differences between the two curves were noticed.

The Standard Curve.—As stated previously, standard curves are designed on the basis of recoveries of thyroxine added to serum. The kinetics of the redox reaction catalyzed by the iodide has been discussed.[5] It was shown that:

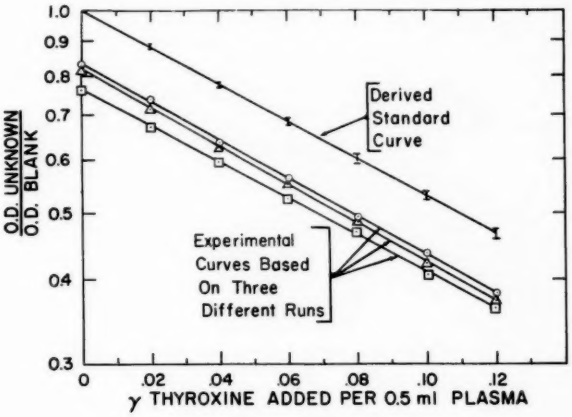
$$\log \left[\frac{\text{O.D. unknown}}{\text{O.D. blank}}\right] = K' [I^-]$$

In plotting optical density ratios at different concentrations of iodide versus the iodide concentrations, a straight line should be obtained, the slope of which equals K′. The optical density ratio of zero thyroxine, i.e., zero iodide, is equal to 1.0, since this optical density ratio is really the optical density blank/optical density blank.

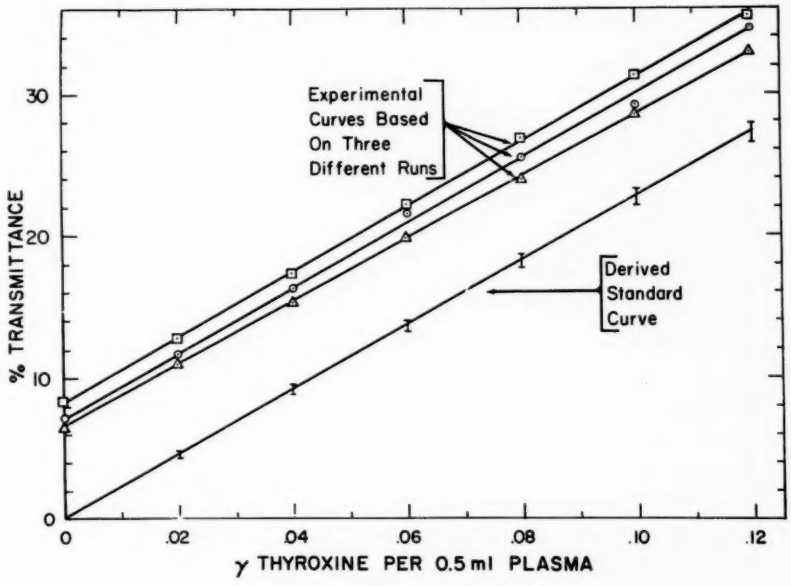
In obtaining the standard curve, Fig. 2, optical density ratios were plotted against concentration of thyroxine added to serum. Hence, the optical density ratio at zero concentration is not equal to that of the blank, but rather to that of the concentration of thyroxine in the standard serum sample. The slope of the experimental curve equals K′ and the line parallel to it, i.e., of slope K′, and intercepting the y axis at the optical density ratio of 1.0 is the true standard curve. The thyroxine concentration in the standard serum can now be read from the derived standard curve against the optical density ratio of the blood sample. To express the abscissa in micrograms per cent of thyroxine, each thyroxine value should be multiplied by 200. To express the abscissa in

*Recent work has indicated that the iodine is most likely reacted with the NaOH to yield iodide and hypoiodite; the latter, because of its instability, is in turn converted to iodate and iodide. The bromide apparently plays no role in this conversion, although its presence seems to stabilize both the reduction and the colorimetric reactions. The use of bromide, therefore, becomes a matter of preference.

micrograms per cent of thyroxine iodide, each thyroxine value should be multiplied by 200 and then by 0.65, since 65 per cent of the thyroxine is iodide, yielding a final correction factor of 130.



A.



B.

Fig. 2.—*A*, Derivation of the standard curve when reading in optical density units. *B*, Derivation of the standard curve when reading in per cent transmittance.

Recovery Studies.—Stock plasma, used to standardize the method, was divided into fractions which were kept in the freezer. Samples of this standard plasma were analyzed daily for a period of about 2 months. No change was found in the thyroxine content of the plasma and the daily variations in the values obtained were negligibly small. Various amounts of thyroxine added to the stock plasma were recovered completely, better than 98 per cent.

Known amounts of thyroxine were added to numerous plasma samples. The values of the added thyroxine ranged from 1 to 40 mcg. per cent. Where the values were outside of the range covered by the standard curve, aliquots of the solutions transferred from the separatory funnels into the colorimeter tubes were taken and analyzed for iodide content after the aliquots were made up to volume with appropriate amounts of KBr and NaOH solutions. For example, if a 1 ml. aliquot was used, 0.8 ml. of 0.03 mg. per milliliter KBr solution and 3.2 ml. 0.25N NaOH were added. In all of these studies, recoveries of better than 98 per cent were effected.

As can be seen in Fig. 2, agreement between different standard curves run on various days is good. One can observe that although the iodide concentrations in the serum samples used in obtaining the standard curves were not the same, i.e., optical density ratios, or per cent transmittance, at zero *added* thyroxine were different, the slopes agree to within 97 per cent. Duplicates or triplicates of identical samples of plasma or serum run in our laboratory have customarily varied by less than 1 per cent.

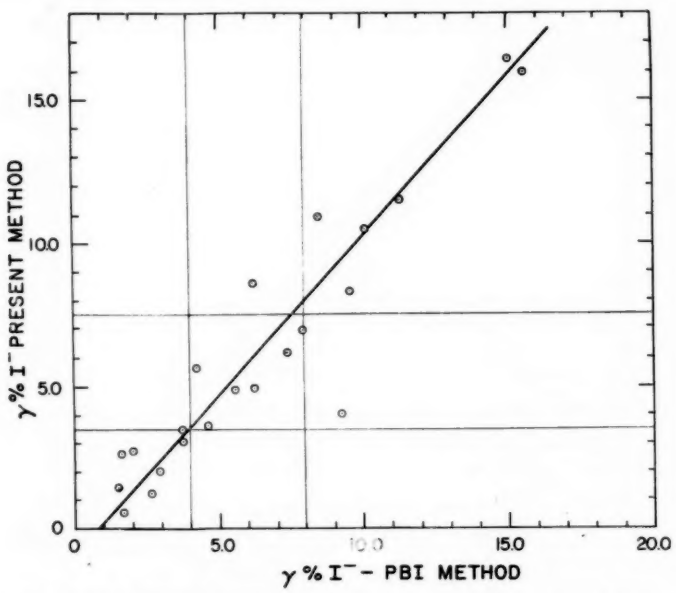


Fig. 3.—A comparison between thyroxine iodide values on clinical blood samples determined by the present method and the protein-bound iodine method.

Comparison With Protein-Bound Iodine.—Fig. 3 shows the comparison between the analysis of clinical samples determined by both the present and the protein-bound iodine method of Barker.[9] The agreement between the two methods is as good as could be expected. On a whole, the values of the present method are somewhat lower than the protein-bound iodine values, in accord with reports from other laboratories.[4]

Conclusions.—There are a number of advantages to the present method over existing methods. The superiority of butanol extraction to serum precipitation is well recognized.[1, 4] However, whereas the butanol has to be

evaporated in the methods of Man and co-workers[3] and of Kontaxis and Pickering,[5] in the present method this step is completely eliminated. As a matter of fact, in the time required for the evaporation of the butanol, the present analysis is complete. No trouble has been encountered with regard to contamination, which is a constant accompaniment of all protein-bound iodine and butanol extractable iodide methods, presumably because all steps are carried on in glass stoppered glassware with minimum exposure to the surroundings. It was convenient to run as many as ten samples in duplicate in a day's work. The average time required for the complete analysis of a blood sample is 6½ hours. The greatest merits of this new method, then, are that it is simple, accurate, and timesaving. This method can be easily modified for determination of thyroxine iodide extracted from tissue with butanol.

SUMMARY

A new method for the determination of butanol extractable iodide has been described. The method is simple, accurate, and short and affords a great degree of reliability. Results obtained by this and by the protein-bound iodine method on clinical samples compared favorably.

I wish to acknowledge the contributions of Dr. John Mason, Chief, Endocrinology Laboratory, Walter Reed Army Institute of Research, and Dr. David A. Hamburg, Chief, Adult Psychiatry Branch, National Institute of Mental Health, without whose cooperation the present work would not have been possible.

I also wish to thank Eugina H. Gregory of the Clinical Pathology Laboratory for supplying the blood samples and the protein-bound iodine analysis.

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STUDIES WITH DOUBLY LABELED IRON

I. Simultaneous Liquid Scintillation Counting of Isotopes Fe^{55} and Fe^{59} as Ferrous Perchlorate

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The simultaneous use of two iron tracers provides an important investigative tool in the fields of biology and ferrous metallurgy. This article describes a precise method for the determination of the isotopes Fe^{59} and the difficultly measured Fe^{55} in the same sample. After conversion of the total iron content to ferrous perchlorate by any suitable means, the sample to be assayed is dissolved in ethanol-toluene scintillation fluor and counted directly in a liquid scintillation spectrometer. High counting efficiencies and technical simplicity are obtained with the method.

METHODS utilizing doubly labeled iron have wide investigative applicability in both biologic[1-3] and metallurgic fields. Both Fe^{59} and Fe^{55} have suitable decay rates for use as tracers, with approximate half-lives of 46 days and 2.9 years, respectively. Fe^{59} is quantitatively determined rather easily, having an average beta particle energy[4] of 0.12 mev., one component (54 per cent) having a maximum energy of 0.46 mev., and the other (46 per cent) 0.27 mev. Associated gamma radiations have energies of 1.1 and 1.3 mev. Fe^{55} is difficult to measure since its most energetic emission is an x-ray of only 0.0059 mev., occurring in approximately 25 per cent of the disintegrations. The other 75 per cent of events result in the emission of low energy electrons and L and M x-rays which, for practical purposes, are not measurable by currently available means.

In 1946, Peacock and collaborators[1] developed two Geiger-Müller tubes, one of which is sensitive to Fe^{55} and the other to Fe^{59} with minimal cross response. The counting efficiency of Fe^{55} by this method is reported to be of the order of 0.5 to 1.5 per cent. Preliminary studies[5] in this laboratory indicated that liquid scintillation techniques could be applied to the measurement of the isotopes with the advantages of increased counting efficiency and utilization of standard, commercially available counting equipment.

MATERIALS AND APPARATUS

Reagents.—

1. Ascorbic acid reagent. Each 3 ml. aliquot of this reagent contains 8 mg. of ascorbic acid, 0.08 ml. of distilled water, made up to a total volume of 3 ml. with absolute ethanol (prepared daily).

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2. Toluene scintillation mixture. This contains 4 Gm. of PPO(diphenyloxazole, scintillation grade*) and 0.050 Gm. of POPOP(1,4-bis(2-(5-phenyloxazolyl))benzene,* scintillation grade) in 1 L. of toluene.

3. Isotopes were obtained in the form of sterile solution of ferrous sulfate, pH 4.†

4. Ferrous perchlorate, hexahydrate, reagent.

5. Perchloric acid, 0.4M.

Apparatus.—

1. Syringe type pipettes[6]‡ of appropriate size are used for all reagent deliveries. The filling attachment available with the device has not been tested. Use 20 gauge needle as orifice.

2. Counting vials, 20 ml. low activity glass with polyethylene cap.§

3. The Tri-Carb liquid scintillation spectrometer‖ is used for all measurements. The temperature of the refrigeration unit is maintained at 8° C.

Preparation of Samples, Standards, and Blanks.—

Samples: The total iron content of an aliquot of the material to be analyzed for activity is converted to ferrous perchlorate by any suitable means. In most of the following experiments, the iron was isolated from doubly labeled blood specimens with a method described elsewhere,[7] but in a few studies, special samples were prepared by adding isotope to weighed amounts of reagent ferrous perchlorate. Although the size of the aliquot will depend on the specific activity of the material to be analyzed, the procedure outlined below is based on a sample containing a total of 10 mg. of iron. Because of differences in counting efficiency between the two isotopes, an attempt should be made to adjust experimental conditions so that the Fe^{55} content of samples will be of the order of five times greater than the Fe^{59} content. In this way, the absolute counting rates will be approximately equal and cross counting errors minimized.

Standards: Standards are prepared from aliquots of ferrous perchlorate containing known amounts of each isotope separately. The final counting mixture for standards should have the same composition as samples and, therefore, both should be prepared by the same procedure. In the experiments described, standards were prepared with iron separated electrolytically[7] from ferric chloride solutions of known specific activity.

Blanks: Blanks for background determination are prepared by adding 3.0 ml. of ascorbic acid reagent to 13.5 ml. of toluene scintillation fluor.

METHODS

Procedure.—For samples containing 10 mg. of iron. (1) add the measured amount of ferrous perchlorate to a counting vial; (2) add 3.0 ml. of ascorbic acid reagent and swirl until mixed and colorless; (3) add 13.5 ml. toluene scintillation fluor, cap vial, and mix by repeated inversion; (4) cool vial 2 hours in scintillation counter refrigeration unit at 8° C.; (5) determine counting rate of sample, standard, and background at tap Y and tap Z, where taps Y and Z represent the photomultiplier voltage setting for optimal counting of Fe^{59} and Fe^{55}, respectively; (6) after determining net counting rate (counts per minute) by subtracting the appropriate background rate, calculate the cross counting constant (K) from the Fe^{59} standard as follows:

$$K = \frac{\text{c.p.m. tap Z}}{\text{c.p.m. tap Y}}$$

*Chemicals available from Tracerlab, Inc., Boston, Mass., (LSP-2 and LSP-7) or from Packard Instrument Co., La Grange, Ill.

†Abbott Laboratories, North Chicago, Ill.

‡Made by Becton, Dickinson and Company, Rutherford, N. J.

§Wheaton Glass Co., Millville, N. J.

‖Packard Instrument Co., La Grange, Ill.

(7) Calculate activity of samples (and mixed standards if used):

$$Fe^{59} \text{ activity} = \text{c.p.m. tap Y}$$
$$Fe^{55} \text{ activity} = \text{c.p.m. tap Z} - (\text{c.p.m. tap Y} \times K).$$

Determination of Counter Settings.—All counts are made with discriminators A and B set at 10 and 70 volts, respectively. This window width should be approximately the same with other Tri-Carb spectrometers. It is necessary, however, to determine the optimal photomultiplier voltage for the individual counter since this is a characteristic of the phototubes. In Fig. 1, the counting efficiency for each isotope is plotted as a function of photomultiplier voltage with a 10 to 70 volt window. The optimal voltage for the determination of each isotope (tap Y for Fe^{59} and tap Z for Fe^{55} is a compromise point where the counting rate for the isotope is maximal and where negligible or acceptably low cross counting of the other isotope is detected. Cross counting should be no greater than 10 to 15 per cent. The reader is referred to the work of Okita and co-workers,[8] who have described in detail various procedures for optimizing counter settings, and the use of the discriminator-ratio method of counting when an automatic sample changer is used. Occasional cleaning of the phototube faces with a soft tissue is essential. Otherwise, a significant decrease in efficiency of counting of Fe^{55} and increase in cross counting fraction may occur.

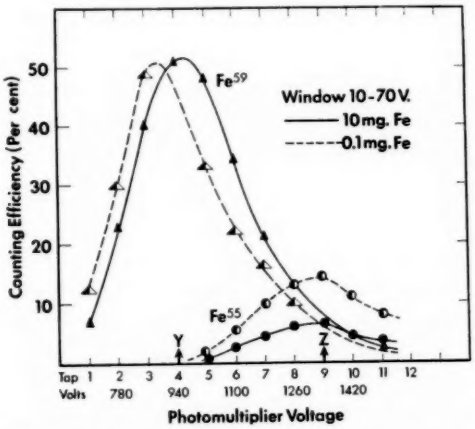


Fig. 1.—Counting efficiency for Fe^{55} and Fe^{59} as a function of photomultiplier voltage in a liquid scintillation system. Counting efficiency is expressed as counts observed, divided by the calculated disintegrations per minute times 100. Broken lines, Isotope contained in 0.1 mg. of carrier iron as reagent ferrous perchlorate. Solid lines, Isotope in 10 mg. of carrier iron as ferrous perchlorate prepared by described process. Composition of counting fluid is given in Table I.

Evaluation of Method.—In order to determine the effect of the amount of iron on counting efficiency, it was first necessary to define the optimal composition of the counting fluid for various amounts of ferrous perchlorate containing from 0.1 to 20 mg. of iron. Isotope was added to the stable solutions in volumes of 0.002 ml. in order to minimize the addition of water to the solution. A detailed study of the outlined procedure for a 10 mg. sample of iron prepared by the electrolytic process was carried out by evaluating the effect of variation of each of the constituents of the final counting mixture on the counting rate. In each experiment, only one constituent or technique was varied, the others remaining as recommended in the method. Reference samples, each containing 10 mg. of iron, were prepared by the electrolytic method from ferric chloride and processed exactly according to the outlined procedure. In all cases, results are expressed as per cent variation from a reference sample, i.e., the difference between the observed counting rates of the experimental and the reference expressed as per cent of that of the reference sample. A few studies were made of the background counting rates for blanks of various composition.

Reliability was assessed by carrying a series of electrolytically processed blood samples and standards, each containing an identical amount of isotope, and 10 mg. of iron through the procedure as recommended.

RESULTS

Effect of Amount of Iron on Counting Efficiencies.—The composition of the counting fluid for various amounts of iron is presented in Table I. Maximal counting efficiency will be obtained with the preparations listed in Table I, part A. The volumes of absolute ethanol, a known quenching agent, given in this section of the table, approach the least amount capable of holding reagent ferrous perchlorate in toluene fluor as a single liquid phase system at 8° C. A small amount of ascorbic acid, roughly proportional to the amount of iron, was necessary to decolorize the mixture to water whiteness. In contrast, the compositions listed in Table I, part B, represent suggested practical conditions for electroplated iron. These preparations will have a high technical safety factor from the point of view of stability and solubility but will exhibit some sacrifice in counting efficiency.

TABLE I. COMPOSITION OF COUNTING MIXTURE FOR VARIOUS AMOUNTS OF IRON PERCHLORATE

IRON IN SAMPLE (MG.)	ADDED CONSTITUENTS						TOLUENE SCINTILLATION FLUOR (ML.)
	A. FOR MAXIMAL COUNTING EFFICIENCY*		B. SUGGESTED PRACTICAL COMPOSITION†				
	ETHANOL (ML.)	ASCORBIC (MG.)	ETHANOL (ML.)	PERCHLORIC ACID 0.4M‡ (ML.)	WATER ADDED (ML.)	ASCORBIC ACID (MG.)	
0.1	0.3	0.8	—	—	—	—	13.5
1.0	0.5	1.3	0.5	0.2	0	1.0	13.5
5.0	1.0	2.7	2.0	1.0	0.04	4.0	13.5
10.0	1.5	4.0	3.0	2.0	0.08	8.0	13.5
20.0	2.5	6.7	3.5	3.0	0.12	16.0	13.5

*Iron as reagent ferrous perchlorate.

†Iron prepared by rapid electroplating procedure; constituents adjusted for positive stability.

‡Volume of perchloric acid used to dissolve electroplated iron before evaporation of excess water.

Efficiency curves were determined for each isotope and for each amount of iron with system components adjusted as indicated in Table I. Examples of these curves for the particular instrument used are presented in Fig. 1. The efficiency curves represented by the broken lines were obtained with ferrous perchlorate samples containing 0.1 mg. of iron. The solid curves, prepared from samples containing 10 mg. of electroplated iron and constituents indicated in Table I, part B, demonstrate classic quenching phenomena: a shift to the right of the curves, and reduction of peak counting efficiency, particularly with the weak emitter. The relatively greater shift of the Fe^{59} curve will result in greater cross counting of Fe^{59} at tap Z but the effect is not large.

The maximal (peak) counting efficiencies for each quantity of carrier iron with the counting fluid composition presented in Table I are given in Fig. 2. Within the limits of the experiment, the efficiency for Fe^{59} is relatively independent of the amount of iron in the sample or composition of the mixture. With Fe^{55}, an increase in the amount of iron and associated changes in fluid

composition result in a decrease in counting efficiency for both types of counting mixtures. Efficiencies represented by the broken line approach the maximum obtainable with this method. At the level of 10 mg. of iron, the use of high stability safety factor mixtures (solid line) results in a 35 to 40 per cent decrease in counting rate. Suggestions for reducing this loss will be presented in a subsequent section.

The relatively flat slopes of the Fe^{55} curves in Fig. 2 indicate that, within the limits of the experiment, greater absolute counting rates will result from increasing the amount of iron in the sample. For example, with a specimen of given specific activity, a sample containing 20 mg. of iron will count with one half the efficiency of a 1 mg. sample, but will have 20 times the total disintegrations per minute of the smaller sample. Therefore, increasing the sample size from 1 mg. to 20 mg. will result in a tenfold increase in absolute counting rate.

The results of a few attempts to reduce the amount of carrier iron below 0.1 mg. were erratic, probably because of adsorption of the isotope on the glass walls of the counting vessel. However, in a few studies, counting efficiencies of 14.5 per cent were obtained with Fe^{55}.

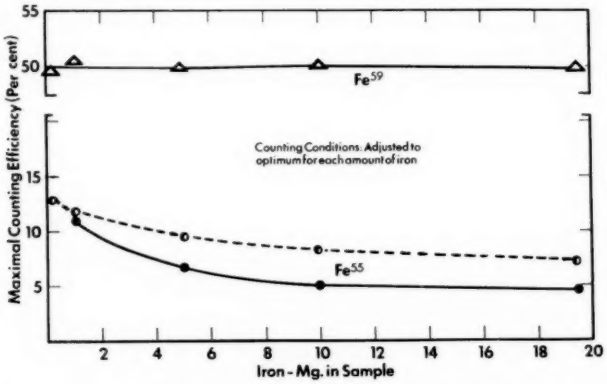


Fig. 2.—Maximal (peak) counting efficiency obtainable with various amounts of carrier iron in sample. Isotope content constant for each amount of iron. Efficiencies were determined at taps Y and Z (Fig. 1) for Fe^{59} and Fe^{55}, respectively. Ordinate, counting efficiency expressed as counts observed, divided by the calculated disintegrations per minute times 100. For Fe^{55}, broken line, reagent ferrous perchlorate, counting fluid, Table I, part A; solid line, electrolytic iron, counting fluid, Table I, part B. For Fe^{59}, composite curve for both salt preparations.

Cross counting constants for samples containing 1, 5, 10, and 20 mg. of iron were 0.09, 0.12, 0.14, and 0.15 for ferrous perchlorate and 0.10, 0.13, 0.15, and 0.17 for electrodeposited iron. The shape of the curves will vary somewhat with different counting instruments, even with properly adjusted electronics.

*Analysis of Procedure for 10 mg. of Electroplated Iron.**—

Iron: The effect of variations in the amount of iron contained in the sample is summarized in Fig. 3. (This study should be differentiated from that presented in Fig. 2, in which a different and optimal counting fluid was used

*All variables are related to "reference sample" defined in Methods.

for each amount of iron.) In the experiments of Fig. 3, the amount of iron alone was varied. For amounts of iron varying from 3 to 12 mg., there is little effect on the counting rate of Fe^{59} or on the cross counting constant. With Fe^{55}, on the other hand, there is a progressive but slight decrease in counting rate with decreasing amounts of iron in the sample (0.2 per cent per milligram iron). Within solubility limits, the method is relatively insensitive to variations in the amount of carrier iron. The amount of total perchlorate (0.8 mM.) is the same for each amount of iron. Titration studies demonstrated that the hydronium perchlorate varies from 0.68 to 0.37 mM. as the iron content of the sample is increased from 3 to 12 mg. From these data, within the range of concentrations studied, it is evident that the relative proportions of ferrous and hydronium perchlorate are of minor significance in affecting the counting rate for Fe^{55}. The lower section of Fig. 3 indicates a fairly constant background counting rate.

Perchloric acid: Studies of the effect of variations in the amount of dilute perchloric acid used to dissolve the electroplated iron are summarized in Fig. 4. Before preparation of the sample, the excess water introduced by the dilute acid is removed by evaporation. Titration studies indicate that little,

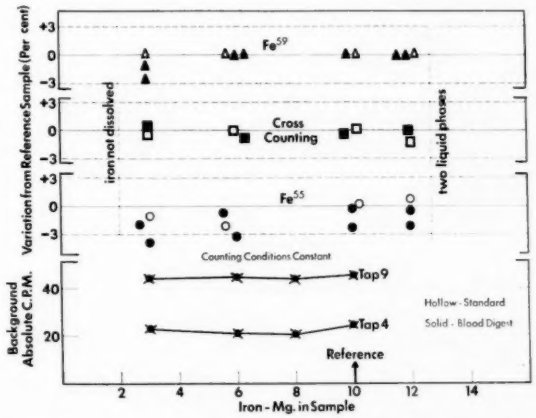


Fig. 3.—Effect of various amounts of electroplated iron in the sample on counting rate and on background. In these experiments, the amount of carrier iron alone was varied; isotope content and other constituents of the counting mixture were constant. Ordinate (upper section): the difference between the counting rates of an experimental and reference sample expressed as a percentage of that of the reference sample.

if any, excess perchloric acid is lost during evaporation, and is, therefore, carried over into the final counting mixture. The data of Fig. 4 indicate that the counting efficiency of Fe^{59} and cross counting are not greatly affected by variations in the amount of perchloric acid used. However, the counting efficiency of Fe^{55} is highly sensitive to this variable (1.5 per cent per 0.1 ml. acid).

Water: It was found that the addition of small amounts of water to the sample resulted in an increase in the counting rate for Fe^{55}, and a decrease in the cross counting constant as illustrated in Fig. 5. It should be noted that the slope of the curve for Fe^{55} is changing rapidly as zero excess water

is approached. When an evaporation step is included in the preparation of ferrous perchlorate, errors due to slight variations in the degree of dryness can be minimized by the addition of water.

Ethanol: The data of Fig. 6 show that progressively increasing amounts of ethanol have little effect on the counting rate of Fe^{59}, but result in a distinct reduction in counting efficiency for Fe^{55} and an increase in cross counting. A variation of 0.1 ml. in the measurement of the volume of ethanol alters the counting rate of Fe^{55} from 1 to 3 per cent.

Ascorbic acid: The addition of ascorbic acid is essential since there is some ferric salt (colored) in most preparations of ferrous perchlorate. Variation in the amount of ascorbic acid from 3 to 16 mg. had little effect on the counting rate of either isotope or the cross counting constant (Fig. 7).

Fig. 4.

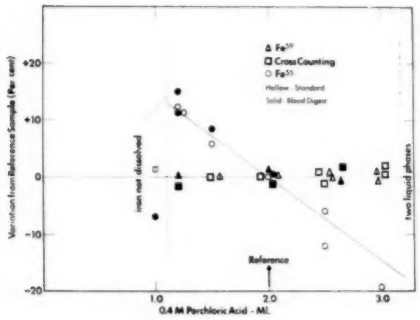
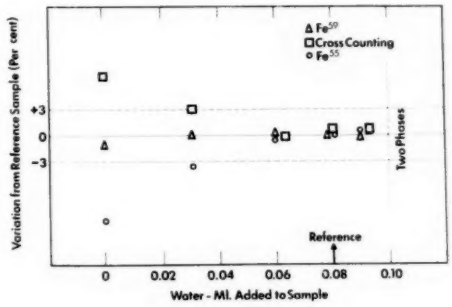
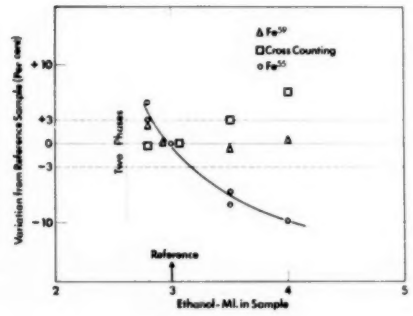


Fig. 5.





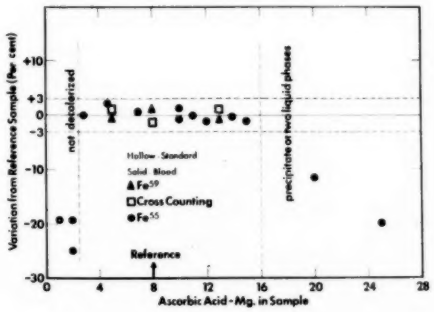


Fig. 6.

Fig. 7.

Figs. 4, 5, 6, and 7.—Effects of variation of the content of each of four constituents of the final counting mixture for 10 mg. of plated iron. In each figure, only one component was varied, the others remaining as outlined in the procedure. Ordinate (for each figure): the difference between the counting rates of an experimental and reference sample expressed as a percentage of that of the reference sample.

Stability: The effect of the age of the sample, measured from the time of placement in the refrigeration unit to the time of counting, on the observed rates is given in Fig. 8. From the data it is evident that counting should be accomplished between 2 and 24 hours, since maximum stability occurs in this interval. Although there is a progressive increase in counting rate of Fe^{55} with age, both standards and samples are similarly affected. Three examples of deterioration effects are included in Fig. 8, viz., a decrease in the

counting rates of both Fe^{55} and Fe^{59} or a variation in the cross counting constant in either direction. The earliest evidence of deterioration may be an alteration in the cross counting constant. Deterioration of a sample before 3 to 4 days is uncommon and rarely occurs without obvious discoloration, but the exceptional case makes visual inspection for color change unreliable as an index of stability. A few long-range stability studies (1 to 2 weeks) indicated that stability was improved by an increase in the amount of ascorbic acid. It was also noted that samples prepared from blood digestates by the electrodeposition method were far less stable than those prepared from ferric chloride by the same technique.

Blanks: A comparison was made of background counting rates of samples prepared from both nonradioactive blood digestates and standards containing from 3 to 12 mg. of iron, from a mixture of 3 ml. of ascorbic acid reagent or absolute ethanol in toluene fluor, and from 13.5 ml. of toluene fluor alone. In all cases, the background counting rates were not different from the rates presented in Fig. 3.

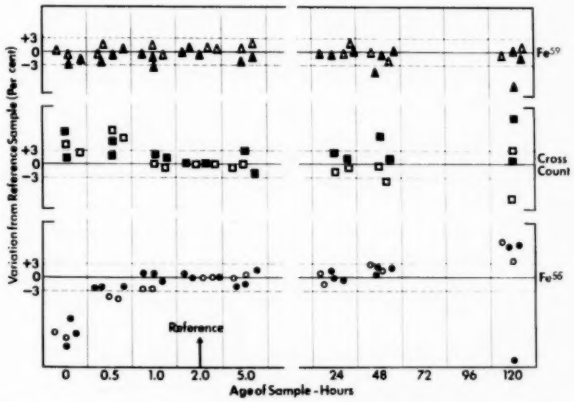


Fig. 8.—Stability of the sample. Abscissa: age of sample measured from time of placement in refrigeration unit. Ordinate: the difference between the counting rates of an experimental and reference sample expressed as a percentage of that of the reference sample. Data are corrected for isotope decay.

Reliability.—In order to evaluate the precision of the method, an identical amount of Fe^{59} (15,000 c.p.m.) was added to each of a series of seven samples, and a similar series prepared with Fe^{55}. Single, one minute counts were made. The mean cross counting constant was 0.149; for Fe^{59}, the coefficient of variation $\pm$ 0.696 per cent, for Fe^{55}, $\pm$ 0.968 per cent, and for the cross counting constant, $\pm$ 0.992 per cent. It should be noted that these samples were carried through digestion and electroplating procedures, and therefore, the errors inherent in the chemical operations as well as those of the counting procedure are included.

DISCUSSION

In a previous report,[5] the reasons for selecting an ethanol-toluene system for counting was described in detail. Subsequent experience indicated that the perchlorate method offers a marked increase in counting efficiency and

stability over the previously described trichloracetate procedure. However, it is recognized that the development of an easily prepared, toluene soluble, colorless, iron chelate would offer a great technical advance.

The maximal counting efficiency of Fe^{55} obtainable with this method is of the order of 14 to 15 per cent. It should be recalled that practicably measured emanations from Fe^{55} occur in only 25 per cent of the total disintegrations. Of these 60 per cent can be recorded by this method.

The conditions recommended in the procedure (reference in each of the figures) offer a wide margin of technical safety. It is estimated, from a summation of the individual data that an increase of at least 30 per cent in counting rate for Fe^{55} could be achieved by reducing the safety factor to a minimum. Although not studied in detail, the amount of ethanol can be further reduced by increasing the temperature of the refrigeration unit. However, the temperature used is not far from the maximum compatible with good instrumentation.

SUMMARY

1. A procedure is described for the simultaneous quantitative measurement of the isotopes Fe^{55} and Fe^{59} as ferrous perchlorate by liquid scintillation counting techniques.

2. With this method, maximal counting efficiency is of the order of 50 per cent for Fe^{59} and 14 per cent for Fe^{55}. The latter figure represents a detection of 60 per cent of the practicably measured emanation of Fe^{55}.

3. The counting rate for Fe^{55} was depressed by increases in the content of ethanol and total perchlorates in the mixture, but only slightly affected by the proportion of ferrous and hydronium perchlorate. Quenching was not observed with Fe^{59}.

4. A study of the effect of variations in the composition of the counting mixture is presented.

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